Generation of Mycophenolate Mofetil Resistant Lymphocytes for Adoptive Immunotherapy

---

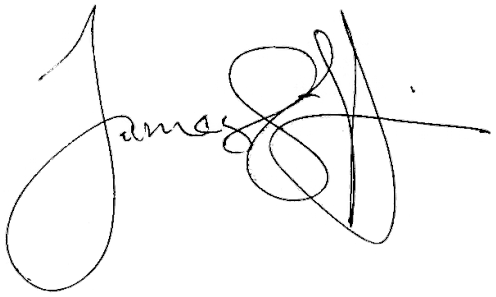
James Edward Griffin

A thesis submitted to
University College London (UCL)
for the degree of

DOCTOR OF PHILOSOPHY

Declaration

I, James Edward Griffin confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.



Dedication

This work could not have been completed without help from many people. To the laboratory technicians, masters and PhD students and post-doctoral fellows who both made the laboratory a friendly place to be and offered help and advice, I would have not made it to the end of my studies without you. I thank the Wellcome trust for funding this work and to Ron for believing, supporting, advising and guiding me throughout this project. By far the most important thank you is to my family. My father let me move back home, with my wife and initially one and then two sons. Without his support I would not have been able to start this project. Most importantly, my wife and sons who moved across the country for me and then spent every day (including weekends), for most of 2 years, saying goodbye while I went to work. Thank you.

Abstract

Background

Adoptive transfer of cells targeting viral infection or tumour following allogeneic transplantation is hindered by the requirement for immunosuppression. Reports of engineered resistance to calcineurin inhibitors have highlighted the potential of conferring immunosuppressive resistance in this setting. A commonly used immunosuppressant, mycophenolate mofetil (MMF) is a non-competitive inhibitor of inosine-5'-monophosphate dehydrogenase (IMPDH). I used T cells transduced with mutated IMPDH that confers >2000-fold resistance to MMF ($IMPDH2^{R}$; T333I, S351Y).

Methods

$IMPDH2^{R}$ and IMPDH2 with a catalytic site mutation ($IMPDH2^{CS}$; C331A) were cloned into retroviral vectors as fusions to eGFP reporter sequences. T cells were transduced with either vector, transferred to recipient mice and evaluated in the presence or absence of MMF.

Results

A selective advantage for $IMPDH2^{R}$ was demonstrated using a 1:1 mix of CD8 T cells transduced with $IMPDH2^{R}$ and $IMPDH2^{CS}$. These were injected into recipients distinguishable from the transferred cells using congenic markers. Transferred cells were stimulated by cognate peptide and MMF (0-200mg/kg/day) was given daily. Selection of $IMPDH2^{R}$ transduced cells occurred following stimulation even without drug (Mann-Whitney Ratio

IMPDH2^{R}:IMPDH2^{CS} p=0.0356). MMF administered significantly increased selection (Mann-Whitney MMF 0 v 200 p=0.0365).

To assess function, irradiated mice were injected with EL4.OVA tumour cells subcutaneously. After 10 days, either MMF or vehicle treatment was initiated and OT1 CD8 T cells transduced with either IMPDH2^{CS} or IMPDH2^{R} transferred. The combination of MMF and IMPDH2^{R} transduced cells resulted in significantly improved survival over vehicle treated mice (log rank p<0.0001) with significantly smaller tumours at day 26 (Mann-Whitney p<0.0001). This effect was due to direct synergistic effects of both MMF and transferred T cells against tumour cells.

Conclusions

IMPDH2^{R} confers cells with a selective advantage. Synergy with MMF resulted in improved tumour survival. In patients requiring on-going immunosuppression during adoptive immunotherapy, this combination has great therapeutic potential.

Index

Tables

Figures

Abbreviations used

6-MP	6-Mercaptopurine
7-AAD	7-Aminoactinomycin D
ADP	Adenosine diphosphate
ALL	Acute Lymphoblastic Leukaemia
AMP	Adenosine Monophosphate
APC	Antigen presenting cell
ATG	Antithymocyte globulin
ATP	Adenosine triphosphate
BMT	Bone Marrow Transplant
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
BW	BW5247 thymoma cell line
CAR	Chimeric Antigen Receptor
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CLL	Chronic Lymphocytic Leukaemia
CMV	Cytomegalovirus
ConA	Concanavalin A

CTL	Cytotoxic T Lymphocyte
dADP	Deoxyadenosine disphosphate
dGDP	Deoxyguanosine diphosphate
dGTP	Deoxyguanosine triphosphate
DLI	Donor lymphocyte infusion
DNA	Deoxyribonucleic acid
EBV	Epstein Barr Virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked ImmunoSorbant Assay
FACS	Flow cytometry
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
GvHD	Graft versus Host Disease
GvL	Graft versus Leukaemia
HGPRT	Hypoxanthine-guanine phosphoribosyltransferase
HLA	Human Leukocyte Antigen
HSCT	Haematopoietic Stem Cell Transplantation

IFA	Incomplete Freund's adjuvant
IFN	Interferon
IL	Interleukin
i.p.	Intraperitoneal
i.v.	Intravenous
IMP	Inosine Monophosphate
IMPDH	Inosine-5'-monophosphate dehydrogenase
K_i	Dissociation constant
LCL	Lymphoblastoid cell line
LMP	Latent membrane protein
MACS	Magnetic-activated cell sorting
MFI	Median fluorescence intensity
MHC	Major Histocompatibility Complex
MMF	Mycophenolate Mofetil
MPA	Mycophenolic acid
MPAG	Mycophenolic acid glucuronide
mRNA	Messenger ribonucleic acid
MSC	Myeloid suppressor cells
MTOR	Mammalian target of rapamycin (Also known as mechanistic target of rapamycin)

NAD	Oxidised nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NK cell	Natural Killer cell
NO	Nitric oxide
OVA	Ovalbumin
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium Iodide
PRPP	5'-phosphoribosyl-1-pyrophosphate
PTLD	Post-transplant Lymphoproliferative Disorder
RNA	Ribonucleic acid
s.c.	Subcutaneous
SOT	Solid Organ Transplantation
TAP	Transporter associated with protein
TBI	Total Body Irradiation
TCR	T cell receptor
Tg	Transgenic
T_h cells	T helper cells

TLR	Toll-like receptor
TNF	Tumour necrosis factor
T_{Reg}	Regulatory T cells
XMP	Xanthine Monophosphate
ZFN	Zinc finger nuclease

Chapter 1: Introduction

Background

The effector cells of the adaptive immune system are activated in a targeted fashion when T cell receptors (TCR) on the lymphocyte cell surface bind to specific antigens presented by host major histocompatibility (MHC) molecules. Following recognition and lymphocyte activation, cytolytic activity is targeted towards cells presenting 'foreign' antigen such as virally derived antigens on infected cells or neo-antigens on tumour cells. Lymphocytes possessing a diverse repertoire of TCRs are generated but negative selection within the thymus removes lymphocytes expressing TCRs most likely to cause autoimmune disease while retaining cells with potential to respond to foreign antigen. This ability to act in such a specific, targeted fashion makes the use of lymphocytes an attractive therapeutic option for the treatment of malignancy or viral infection.

All TCRs expressed by a single T cell are of the same specificity, directed to a specific combination of human leukocyte antigen (HLA) molecule and antigen. CD8 T cells express TCR specific for HLA class I molecules presenting peptides from cytosolic proteins. Class I molecules are found on the surface of all cells except red blood cells and platelets (Natali et al., 1984). Normal cells express peptides processed from self-antigen to which the CD8+ T cell repertoire is tolerised during negative selection within the thymus (Palmer, 2003). Cells infected by virus will also present viral peptides and tumour cells

may present peptides from neoantigens. These can be recognised by CD8 T cells as foreign, resulting in a cytotoxic immune response.

Whilst not as specific as the use of lymphocytes alone, the benefit of allogeneic stem cell transplantation in treating malignancy is due to the response of lymphocytes from the donor against malignant cells known as graft-versus-leukaemia (GvL). This immunological action works in combination with the conditioning regimen of chemo-radiotherapy with the aim of eradicating disease. GvL effects of lymphocytes are clearly demonstrated in patients with chronic myeloid leukaemia, who have been successfully treated with infusions of donor lymphocytes following relapse post stem cell transplant (Kolb et al., 1990). Non-myeloablative conditioning regimens where less intensive conditioning has enabled use of allogeneic transplant in more elderly patients due to reduced toxicity require the graft versus leukaemia effect to work to prevent disease relapse.

GvL is the beneficial immunological effect of allogeneic stem cell transplant, however in addition to recognition of leukaemia cells, lymphocytes from the donor can recognise host cells as 'foreign'. This causes graft-versus-host disease (GvHD), which results in significant morbidity and potentially mortality (Ferrara et al., 2009). To prevent graft versus host disease, immunosuppressive medication is given which inhibits lymphocyte function. The same agents are used to prevent the recipient immune system rejecting solid organ transplants. While immunosuppression has allowed transplantation to become an established treatment modality, it also results in significant side effects. Some viral infections are not cleared following primary infection and are subsequently kept dormant by an intact cellular immune response (Steven,

1997, Sylwester et al., 2005). Immunosuppression enables reactivation of latent viral infections such as cytomegalovirus (CMV) and Epstein Barr virus (EBV), the latter of which can cause lymphoproliferative disorder (PTLD).

CMV is a herpes virus that infects the majority of people; seroprevalence depends on socioeconomic status and is between 45 and 100% of the population (Cannon et al., 2010). Primary CMV disease is usually asymptomatic or produces a mild flu-like illness. It can cause pharyngitis, lymphadenopathy and fever of unknown origin. It is the largest herpes virus producing more than 200 proteins, of which several are involved in immune evasion thereby preventing clearance of virus (Miller-Kittrell and Sparer, 2009). Lifelong latency is established, primarily in myeloid cells (Poole et al., 2011). The size of the cellular response throughout the rest of an infected individuals life is enormous. CD4 and CD8 T cells with broad specificity for CMV increase throughout life accounting for 10-40% of the memory compartment (Ouyang et al., 2003, Sylwester et al., 2005).

Following transplantation, anti-rejection and anti-GvHD regimens can result in reactivation of latent virus as well as increasing the risk of primary disease. Loss of immune containment allows uncontrolled viral replication with potential for disseminated disease and development of life-threatening end organ damage including pneumonitis, colitis and hepatitis. Up to 80% of seropositive bone marrow transplant recipients will reactivate CMV (Ljungman et al., 2011). Current antiviral therapies have significant side effects including renal dysfunction and suppression of bone marrow function. Mortality from CMV pneumonitis remains at over 50% despite these therapies.

Post-transplant lymphoproliferative disorder (PTLD) represents a spectrum of disease primarily arising from the malignant transformation of germinal centre B cells following solid organ transplantation (SOT) or haematopoietic stem cell transplantation (HSCT). The histology ranges from an indolent polyclonal expansion of B cells to the more common aggressive diffuse large B cell and Burkitt lymphoma types. There is an overall incidence of 1-15% following SOT and 0.5-25% following HSCT with the highest incidence following T-cell depletion and aggressive immunosuppression (Shapiro et al., 1988, Swinnen, 2000, Cockfield, 2001, Domingo-Domenech et al., 2001, Opelz and Dohler, 2003). The reported mortality is approximately 50% (Leblond et al., 1995, Newell et al., 1996, Opelz and Dohler, 2003). The pathogenesis results from the reactivation of the oncogenic virus EBV in 60-80% of cases (Allen et al., 2001, Hoshida et al., 2001). Prior to development of PTLD, EBV infected B cells are seen in increased number within the blood and tissues (Stevens et al., 2001, Muti et al., 2003). Within tumour cells the EBV infection is monoclonal (Capello et al., 2003). Several EBV genes expressed during latent infection have transforming activity for B cells (Cohen, 2003, Hsieh et al., 1999, Tanner and Alfieri, 2001, Kuppers, 2003, Thompson and Kurzrock, 2004). PTLD tumour cells express EBV latent proteins, an ideal immunogenic target for immunotherapy.

Monitoring for EBV DNA in the circulation can identify patients at high risk of imminent PTLD. These patients can then be pre-emptively treated by reducing immunosuppression (if possible) and with rituximab, a human-murine chimeric monoclonal antibody that targets the CD20 positive B-lymphocytes in which EBV is found. Once PTLD has developed systemic chemotherapy must be given, causing greater morbidity and mortality.

Current therapy for CMV and EBV reactivation cause significant morbidity particularly due to renal toxicity and bone marrow suppression and therefore alternative therapy, either pre-emptive or acute, would be welcomed. Viral reactivation could be treated effectively by transferring viral-specific lymphocytes, a process called adoptive immunotherapy. It is possible to collect and expand virus specific donor cells (Heslop et al., 1994b, Rooney et al., 1998, Gustafsson et al., 2000, Hale et al., 2008, Merlo et al., 2008) or redirect the specificity of lymphocytes by *ex vivo* insertion of genes encoding a virus specific TCR prior to transfer (Orentas et al., 2001, Mueller et al., 2012). When a patient still requires on-going immunosuppression following transplantation, to prevent rejection or GvHD, transferred lymphocytes will also be supressed, reducing efficacy.

A potential strategy to circumvent the inhibitory effects of immunosuppression is to engineer therapeutic cells with resistance to immunosuppressive drugs *ex vivo* prior to transfer. Here I discuss the current use of adoptive immunotherapy and redirection of lymphocytes through transfer of specific TCRs. I will discuss the actions of immunosuppressive medication and the potential genes to confer drug resistance. I outline the aims of this research, in which I confer resistance to mycophenolate mofetil (MMF) and investigate the action of these resistant cells during MMF therapy in a murine tumour model.

1.1 Adoptive Immunotherapy

Immunotherapy is a term defined as “the treatment of disease by inducing, enhancing or suppressing an immune response” (Dictionary, 2004). Adoptive immunotherapy involves the transfer of T cells to a recipient with the aim of producing a response against target cells, usually malignant or virally infected cells.

Adoptive immunotherapy has several advantages over conventional therapeutic approaches;

1) Specificity – targeting of tumour or virally infected cells via a specific receptor causing fewer side effects.

2) Longevity – Transferred cells can establish immunological memory and persist to control disease long term.

Effective adoptive immunotherapy requires that transferred cells are of the correct specificity and retain their cytotoxic function following transfer. In most settings it is also desirable for transferred cells to engraft and persist providing long-term immunological memory. Repeated administration of adoptive immunotherapy may also be used and if disease is cleared, memory is not an absolute requirement.

1.1.1 Specificity

The specificity of T lymphocytes is determined by their TCR in an HLA restricted fashion. This means that transferred cells need to be HLA compatible with the recipient, i.e. a lymphocyte with a TCR specific for a viral peptide will only work if the recipients HLA type is compatible. There are several options to either collect or engineer a cellular product of the desired specificity:

- Selection and expansion of pre-existing autologous or allogeneic cells on the basis of their T cell receptor specificity (monoclonal or polyclonal)
- Redirection of lymphocytes towards a specific target by introduction of a new TCR specific for the desired target antigen:MHC complex
- Redirection of lymphocytes towards a specific target by introduction of a chimeric antigen receptor specific for cell surface antigens (not HLA restricted)

1.1.1.1 Selection and expansion of cells of required TCR specificity

If a donor is seropositive for a particular virus i.e. previously exposed, they will have generated virus-specific T cells during the infective episode. Following resolution of infection, some of these cells will persist as a long-lived memory population. When polyclonal T cells are incubated with viral peptides, only cells receiving necessary levels of stimulation will persist. This will result in selection of viral reactive cells that expand while alloreactive cells will not receive stimulation and will undergo apoptosis. After donation, therapeutic numbers of cells can be generated using repeated *ex vivo* stimulation with antigen presenting cells (APC) expressing the desired HLA:viral antigen and cytokine support. It is therefore necessary to identify the immunogenic viral antigen and

express that on an APC in the context of MHC in the presence of co-stimulatory molecules.

Immunodominant antigens have been described for CMV (Berger et al., 2009), EBV (Hislop et al., 2007), adenovirus (Leen et al., 2004), Human Herpes Virus 6 (Gerdemann et al., 2013), BK (Blyth et al., 2011) and Varicella Zoster Virus (Blyth et al., 2012). This has allowed the use of overlapping peptide pools covering the whole viral protein rather than live viruses to be used (Trivedi et al., 2005). Optimisation of protocols in terms of both antigen and cytokine support are reducing the length of *ex vivo* culture required and simultaneous stimulation with peptides from several viruses e.g. EBV, CMV and adenovirus has allowed multi-virus cytotoxic T lymphocyte (CTL) lines to be generated simultaneously (Zandvliet et al., 2011, Gerdemann et al., 2012).

More rapid selection strategies are available and under investigation for clinical use. When there is a high frequency of specific T cells e.g. CMV- or EBV-reactive, magnetically labelled peptide multimers bind to the TCR and allow selection of cells. The product will be specific only for the peptide multimer used, but avoids the need for culture with APCs. There is a requirement for both a high precursor frequency and a large volume of blood/apheresis product. Alternatively, selection of cells that produce interferon gamma (IFNγ) after stimulation with viral antigens allows selection of a broad range of virus reactive cells but still requires large donor blood volumes or apheresis product.

1.1.2 Clinical Use of Virus specific adoptive immunotherapy in stem cell transplantation

The potential for adoptive immunotherapy to reconstitute specific immunity following stem cell transplantation has been studied. Reconstitution of anti-viral

immunity to CMV, using specific CTL, was first reported in 1992 (Riddell et al., 1992). Lymphocytes were expanded using donor fibroblasts as the APC and antigen from the AD169 strain of CMV. The sibling donors were HLA-matched and transferred cells did not cause GvHD. Both CD4+ and CD8+ T cell CMV response was required for long-term persistence. Subsequently, several other groups using a variety of production techniques have successfully reconstituted CMV-specific responses in many patients (Walter et al., 1995, Einsele et al., 2002, Peggs et al., 2003). It should be noted that when CMV antigen generated CD4 CTL were transferred alone the level of conferred immunity appeared much reduced with multiple failures and even death (Perruccio et al., 2005). Therefore it can be postulated that a broad polyclonal product of both CD4 and CD8 lymphocytes, which more closely resembles the endogenous response to CMV, may produce the best results.

Therapy for PTLD was initially trialed with unmanipulated donor lymphocytes (DLI) that led to successful treatment in approximately 70% of patients (Doubrovina et al., 2012) but with a high incidence of GvHD (Heslop et al., 1994b, Papadopoulos et al., 1994). Because up to 95% of adults worldwide have been exposed to EBV (Higgins et al., 2007) and up to 5% of their circulating CD8+ T cells target EBV (Bhaduri-McIntosh et al., 2008), it is an ideal target for collection and expansion of specific T cells. By stimulating donor peripheral blood mononuclear cells (PBMCs) with autologous EBV-transformed B cell lines *ex vivo,* EBV-specific, MHC-restricted cytotoxic T cell lines can be generated, without the auto-reactive T cells responsible for causing GvHD. After transfer these cells are well tolerated and successfully control active EBV replication and PTLD (Heslop et al., 1994a, Rooney et al., 1995).

In vivo persistence has been demonstrated by detection of a neomycin resistance gene retrovirally transduced into CTL *ex vivo* prior to infusion (Heslop et al., 2010). The ability to persist led to prophylactic adoptive immunotherapy for high-risk patients following HSCT, which was effective compared to historical controls (Rooney et al., 1998). Adoptive immunotherapy with EBV-specific CTLs is now an established therapeutic option (Gustafsson et al., 2000, Hale et al., 2008, Merlo et al., 2008) and provides safe and effective reconstitution of EBV immunity both preventing and treating EBV-PTLD after T cell depleted stem cell transplant.

1.1.3 Clinical Use of Virus specific adoptive immunotherapy in solid organ transplantation

EBV-PTLD following solid organ transplantation is recipient rather than donor in origin. Therefore autologous (recipient) EBV-CTLs, or HLA-matched allogeneic EBV-CTLs are required.

1.1.3.1 Autologous EBV-CTLs

The feasibility and safety of CTL infusion following SOT has been established, using autologous EBV-CTLs generated from patient blood taken pre-transplant(Haque et al., 1998). Subsequently EBV-CTL lines have been generated from patients receiving immunosuppressive therapy (Khanna et al., 1999, Savoldo et al., 2001) and induced complete response (CR) in a patient with progressive PTLD. Unfortunately, secondary lesions developed in this patient implying lack of functional persistence of EBV-CTLs in the presence of immunosuppression.

Further studies suggest that responses induced using autologous EBV-CTL for adoptive immunotherapy are often partial or temporary (Comoli et al., 2002,

Comoli et al., 2005, Savoldo et al., 2006, Sherritt et al., 2003). When used prophylactically, none of the first 21 patients treated developed PTLD. For treatment ~50% (19/39) achieved complete remission but 41% (16/39) had no response (Merlo et al., 2008).

Following HSCT, transferred cells expand and persist. In contrast, following SOT, expansion appears more limited with levels dropping prior to the next cell infusion. It is possible that infused EBV-CTLs are unable to proliferate significantly due to immunosuppressive therapy in the absence of significant lymphodepletion.

1.1.3.2 Generation of EBV-CTLs from seronegative recipients

Autologous memory EBV-CTLs cannot be collected for *ex vivo* expansion from EBV seronegative SOT recipients. These patients are at risk of developing primary infection with EBV and PTLD whilst immunosuppressed. Alternative expansion protocols using IL-12 and IL-7 (Comoli et al., 2006) or enrichment of activated CD25 positive cells (Savoldo et al., 2002) have enabled the generation of autologous EBV-CTLs in EBV-negative individuals. Generating sufficient numbers of autologous EBV-CTL takes about 10 weeks for each patient. Therefore another approach is to use pre-generated, allogeneic CTL lines from partially HLA matched third party donors.

1.1.3.3 Third party EBV-CTLs

Haque and colleagues have established a bank of 100 EBV-CTL lines that are immediately available (Wilkie et al., 2004). Cells are selected on the basis of the closest HLA match to the patient. Despite HLA mismatches, a phase II trial showed no toxicity or graft failure and responses in 21 of 33 patients refractory to conventional treatment. Of note, immunosuppression was reduced or

stopped in all patients which may have resulted in recovery of the patients EBV immunity as well as allowing transferred cells to function. Transferred cells do not appear to persist and one patient was shown to have antibodies against the mismatched allo-HLA antigens on the transferred cells (Haque et al., 1998, Haque et al., 2007).

1.1.4 Adoptive immunotherapy in non-transplant patients

Adoptive immunotherapy has also been successfully employed in the treatment of patients with malignancy. Cultured autologous EBV-specific T-cell lines were used by Bollard *et al.* to treat EBV-positive Hodgkin's disease (Bollard et al., 2004). The targeted antigens were only weakly immunogenic, consisting primarily of the latent membrane protein (LMP)1 and LMP2 antigens. Despite this low immunogenicity, viral load decreased, demonstrating the biologic activity of the infused CTLs. The CTLs were able to expand by several log *in vivo* and persisted for over 12 months. EBV CTLs were well tolerated, could control B symptoms (fever, night sweats, and weight loss), and had antitumour activity. Five of fourteen patients entered complete remission, one had a partial response and a further five stable disease.

In 1988, Rosenberg *et al.* used autologous tumour infiltrating lymphocytes to treat metastatic melanoma (Rosenberg et al., 1988). Lymphocytes were extracted from freshly resected melanomas and expanded *in vitro*. 20 patients were treated with these tumour-infiltrating lymphocytes and interleukin-2, resulting in regression of tumour in 60% of patients with the effect lasting up to 13 months.

1.1.5 Redirection of Lymphocytes

When the donor is sero-negative i.e. lacking specific immune cells, or following umbilical cord stem cell transplant where donor cells are limited in number and are virus naïve, it is very challenging to select and expand specific T cells for transfer. An alternative option is to engineer cells *ex vivo* to express a specific receptor. Redirection is achieved by insertion of an alternative TCR or a chimeric antigen receptor (CAR) of known specificity (Sadelain et al., 2003). Redirecting the specificity of collected cells towards a specific target in this way also reduces the time needed to generate the required number of therapeutic cells through repeated cycles of expansion.

1.1.5.1 Redirection - TCR transduction

TCR transduction requires transfer of genes encoding the α and β chains of a specific TCR, usually with high avidity for the antigen of interest, into a T cell (Dembic et al., 1986). To ensure stable expression, the introduced gene must be inserted into the cellular genomic DNA. This can be achieved using either a gamma-retrovirus, lentivirus or transposon system. Following transduction, large populations of antigen-specific T cells are generated (Stanislawski et al., 2001, Xue et al., 2005, Morris et al., 2005, Thomas et al., 2007, Hart et al., 2008, Perro et al., 2010, Tsang et al., 2008, Ahmadi et al., 2011).

The therapeutic potential of such gene-modified T cells was shown by Morgan *et al.*, who documented regression of metastatic melanoma in two out of fifteen patients treated with adoptively transferred autologous T cells transduced with a TCR specific for the tumour-associated antigen MART-1 (Morgan et al., 2006).

TCRs specific for viral antigens have been cloned and shown to confer transduced T cells with antiviral specificity. Latent membrane protein 2 (LMP2) represents the immunodominant epitope following EBV infection. It is expressed on EBV-driven malignancies and is therefore a good candidate for adoptive immunotherapy. A HLA-A2 restricted, LMP2-specific TCR has been cloned and optimised for use in redirection of T cells (Hart et al., 2008). This is specific for EBV LMP2 426-434 (CLGGLLTMV) in the context of HLA A*02:01. Human CMV lower matrix protein pp65 contains the immunodominant epitope for CMV. A TCR specific for this epitope, pp65 protein 495-504 (NLVPMVATV), also in the context of HLA A*02:01 has been cloned and optimised for use following retroviral transduction (Schub et al., 2009).

The strategy of redirecting T cells by transducing TCR genes has potential deficiencies and risk of complications. Because the TCR is HLA specific, different TCRs are needed for each different HLA-type. Techniques to identify TCRs of the correct specificity (peptide and HLA specific) have been developed with a much higher throughput, however this can still take some time. A recent technical report highlighted a high-throughput approach whereby rapid identification of TCR sequences from large collections of samples was enabled by capturing and sequencing the TCR variable regions (Linnemann et al., 2013). A bait library was designed that targets each individual variable and joining element within both TCRα and TCRβ loci, resulting in selective isolation of TCR-encoding elements from sheared genomic DNA. Once isolated, paired-end deep sequencing showed that as few as 500 cells were needed to correctly identify a rearranged TCR. Using this approach a library of tumour reactive TCRs from patient material has been generated. The technique is also able to identify TCRαβ pairs in bulk antigen-specific T cell populations and to

assess the TCR repertoire of intratumoral T cell subsets without knowledge of antigen specificity.

Following transduction there is potential for the introduced genes to ‘mis’-pair with endogenous TCR chains. The resultant hybrid TCR is of unknown specificity and has potential to recognise ‘self’ antigen and cause GvHD. To avoid this several genetic optimisations are routinely utilised;

1. The cassette of genes contains 2A DNA sequences between the α and β TCR genes to ensure equal production.
2. The TCR genes are codon optimised to improve efficiency of translation
3. Disulphide bonds introduced to promote binding between the introduced TCR genes.

More recently methods have been described to delete the endogenous TCR thereby removing the possibility of mispairing (Provasi et al., 2012). By utilising zinc-finger nucleases (ZFN) designed to target conserved regions of the TCRβ and TCRα chain genes, endogenous TCR genes are deleted. This was achieved in a stepwise approach where cells were first exposed to adenoviral vectors containing a TCRα chain targeted ZFN. This is transiently expressed (two to three days) during which time the endogenous TCR gene is deleted. Successfully treated cells become CD3 negative allowing sorting five to eight days after transduction. TCRα deleted T cells are then stably transduced with a lentiviral vector containing a TCRα chain gene. This process can then be repeated for the TCRβ chain. Resultant cells express high levels of the transferred TCR and are both functional and highly specific.

1.1.5.2 Redirection – CAR

As previously mentioned, redirection may also be achieved by the use of CARs. The introduced gene is engineered from the variable domains of an antibody heavy and light chain fused by a flexible linker sequence. This is grafted via a hinge and transmembrane region to TCR activation and costimulatory domains, usually CD3-zeta, CD28 and 41BB or OX40. Following ligation of the CAR to its target ligand, both a 'signal 1' and 'signal 2' are generated resulting in activation of the T cell with resultant cytokine production and lytic activity. Redirection with CARs has several advantages over TCR transduction;

- the inserted gene is shorter and therefore easier to transduce
- there is no mis-pairing of introduced TCR chains with endogenous TCR chains
- the target is HLA independent, therefore one CAR will work in all patients

CARs are only specific for cell surface target molecules and therefore will not be able to be used in the treatment of malignancies where the neoantigen is intracellular and targeted by TCR via presentation by HLA class 1 molecules.

The first clinical trial of CAR-expressing T cells was in ovarian cancer, the target was the α-folate receptor (Kershaw et al., 2006), but no tumour regression was seen. Subsequently the design of CAR genes has changed significantly, particularly in the use of different and multiple co-stimulatory domains and T-cell-activation moieties. Much of this has been in the evaluation of CARs targeting CD19, a B-cell antigen (Kochenderfer et al., 2009, Kochenderfer et al., 2010b, Cheadle et al., 2005, Cheadle et al., 2010, Brentjens et al., 2003, Cooper et al., 2003, Rossig et al., 2006). CD19 is an

appealing target because it is uniformly expressed by the vast majority of B-cell malignancies (Nadler et al., 1983) and in normal tissue restricted to mature B cells, B-cell precursors, plasma cells and possibly follicular dendritic cells.

CD19 CAR T cells have been used in the treatment of chronic lymphocytic leukaemia and follicular lymphoma with some patients achieving complete responses (Brentjens et al., 2010, Brentjens et al., 2011, Kochenderfer et al., 2012, Kochenderfer et al., 2010b, Kochenderfer et al., 2010a, Kalos et al., 2011, Porter et al., 2011).

More recently CD19 CAR transduced T cells have been demonstrated to have clinical activity in the treatment of acute lymphoblastic leukaemia (ALL). CAR T cells were successfully used to treat two children with relapsed refractory pre-B-cell ALL (Grupp et al., 2013). Both patients developed B-cell aplasia and cytokine-release syndrome requiring admission to intensive care. One child remains in complete remission over two years following treatment. Unfortunately the other patient relapsed 2 months after treatment with an escape variant that was CD19 negative. These results have resulted in intense study of CD19 CAR transduced T cells in the treatment of ALL with at least 30 clinical trials in progress at the time of writing.

1.1.6 *In vivo* Expansion and Engraftment - Lymphodepletion

Selection of cells with the correct specificity or redirection of cells by insertion of specific receptors is the first step to effective adoptive immunotherapy. It is also necessary for cells to expand in number and engraft within the recipient. Initial reports of adoptive immunotherapy in metastatic melanoma showed that *in vivo* persistence of cells was very short and clinical responses not sustained (Rosenberg et al., 1990).

Use of immunodepleting conditioning chemotherapy and high-dose cytokine administration increase the efficacy of transferred cells significantly (Dudley, 2002, Dudley, 2005). In clinical trials, a non-myeloablative regimen of cyclophosphamide and fludarabine has been used with additional total body irradiation (TBI) given to some patients. Following cell transfer, patients receive high-dose IL-2 for two to three days. Under these conditions, transferred cells expand and can account for up to 75% of peripheral blood CD8+ T cells six to twelve months after transfer. While not randomised, additional lymphodepletion in the form of TBI increased the response to adoptive immunotherapy with responses correlated with TBI dose (Dudley et al., 2008). The mechanisms by which lymphodepletion results in increased expansion and engraftment of transferred cells are discussed below and summarised in figure 1.1.

Figure 1.1 Mechanisms underlying the impact of lymphodepletion on adoptively transferred T cells

A Adoptive Immunotherapy in a lymphoreplete host may be inhibited by:

a) Competition for antigen at antigen-presenting cells (APCs) and inefficient lymphocyte activation in the absence of co-stimulation

b) Reduced availability of activating cytokines by cellular 'sinks'

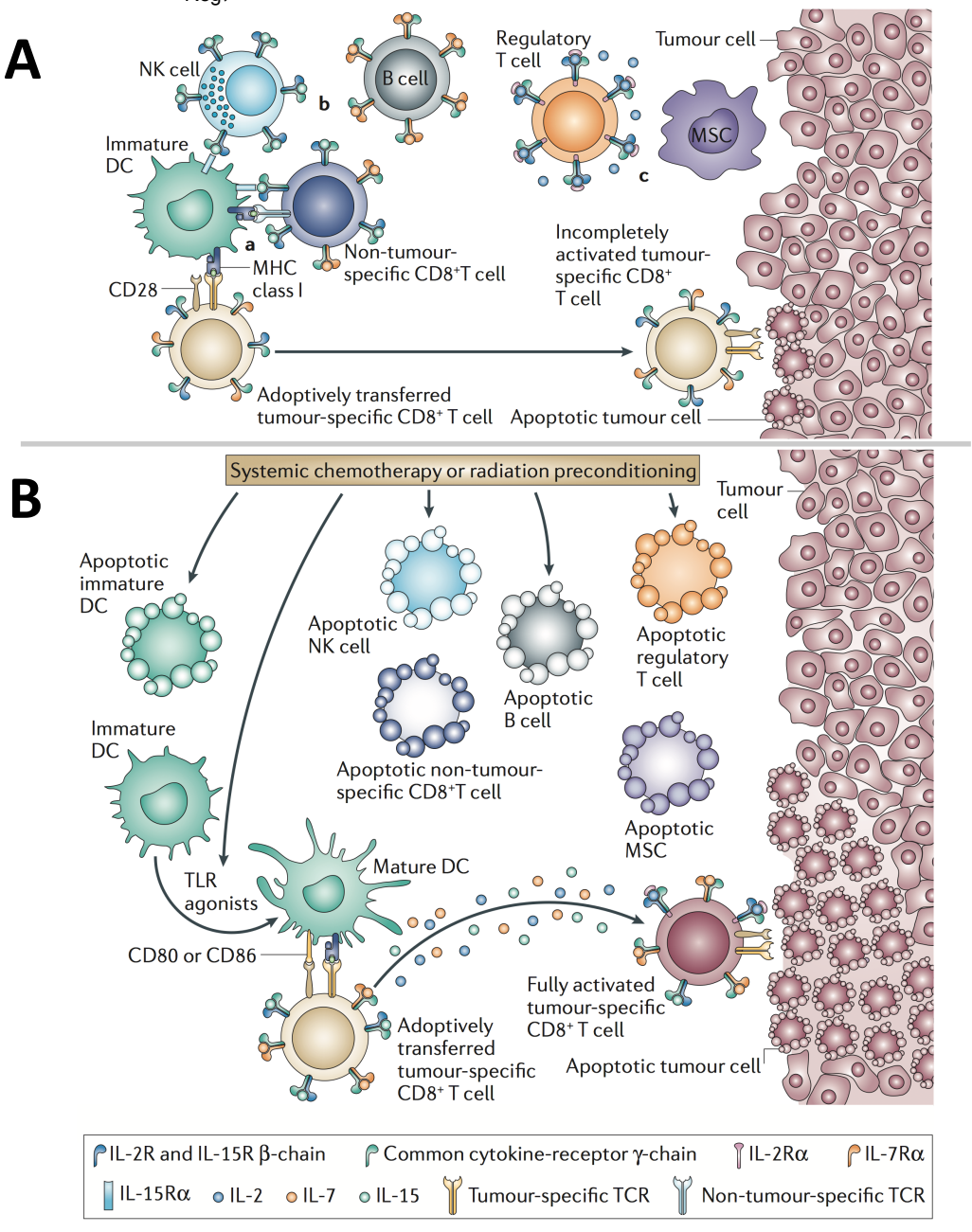
c) Suppressive activities of regulatory T (T_{Reg}) cells, myeloid suppressor cells (MSCs) and possibly Natural Killer (NK) cells

B Prior systemic chemotherapy or radiation may overcome this by:

a) Reducing competition for antigen at APC and activating dendritic cells

b) Removal of cellular 'sinks'

c) Removal of T_{Reg}, MSCs and NK cells



Adapted from: Adaptive Immunotherapy for Cancer: Building on Success
Gattinoni *et al* Nature Reviews Immunology 2006: 6 (5); 383-393

1.1.6.1 Lymphodepletion – elimination of regulatory T cells (T_{Reg})

T_{Reg} cells are a CD4+ population of lymphocytes that are crucial in maintaining peripheral tolerance to self-antigen through suppression of effector cells (Walker et al., 2003, Fisson et al., 2003, Bluestone and Abbas, 2003, Asano et al., 1996). One of the main theories to explain the benefit of lymphodepletion prior to adoptive cell transfer is that elimination of endogenous regulatory T cells removes suppression of the transferred cells. Antony *et al* (Antony et al., 2005) showed that following lymphodepletion, co-transfer of CD8 T cells with CD4+CD25+ T_{Reg} prevented effective adoptive immunotherapy. They used a murine model of adoptive immunotherapy using a subcutaneous B16 tumour (a murine melanoma) to which lymphocytes from pmel-1 TCR transgenic mice are targeted. In Cd4-/- recipient mice, but not Cd8-/-, the anti-tumour effect of transferred CD8 T cells was enhanced. The enhanced anti-tumour was lost if either unselected CD4, which will include T_{Reg}, or selected T_{Reg} were co-transferred with the CD8 T cell effectors. When CD4 T cells depleted of T_{Reg} were co-transferred with the CD8 T cells, there was enhancement of the anti-tumour effect and significant autoimmunity.

We can infer from these data that T_{Reg} cells inhibit the action of effector T cells against tumour; this finding is not surprising as several groups have reported that depletion of T_{Reg} cells enables endogenous immune cells to protect from tumour (Shimizu et al., 1999, Sutmuller et al., 2001, Golgher et al., 2002, Jones et al., 2002). Other cell subsets may be involved in suppression of transferred lymphocytes in lymphoreplete hosts including NK, NK-T and myeloid suppressor cells.

1.1.6.2 Lymphodepletion - Cytokine sinks and homeostatic expansion

The beneficial effect of CD4+CD25- Th cells shown by Antony *et al.* was abrogated when IL-2 deficient CD4 Th cells were used, suggesting provision of cytokine support to CD8 T cells was required (Antony et al., 2005). Cytokine support from Th cells was superior to exogenously administered IL-2, which is commonly used in clinical trials. Lymphocyte number and activation state is regulated by homeostatic cytokines. These include the common gamma chain cytokines IL-2, IL-7 and IL-15. Cytokine availability is essential for both the normal maintenance and proliferation of memory CD8 T cells with IL-7 or IL-15 deficiency causing reductions in the number of memory cells (Schluns et al., 2000, Ku, 2000, Tan, 2002) and over-expression causing memory CD8 expansion (Kieper, 2002, Marks-Konczalik et al., 2000). When cells are transferred in the absence of lymphodepletion, endogenous lymphocytes will compete for the limited amount of cytokine. It is therefore hypothesised that in addition to removal of regulatory cells, lymphodepletion will also remove competition for cytokines.

Following the induction of lymphopenia, spontaneous expansion of any persisting peripheral lymphocytes restores the size of the T cell pool (Mackall et al., 1997). Homeostatic proliferation of lymphocytes is due to recognition of self-MHC/peptides in a process similar to thymic positive selection (Marrack et al., 2000, Surh and Sprent, 2000, Goldrath and Bevan, 1999, Ernst et al., 1999). When lymphocytes are transferred into a lymphopenic host, they also undergo homeostatic proliferation, the rate of which is reduced in a dose-dependent manner depending on the number of transferred cells (Cho et al., 2000, Dummer et al., 2001).

Dummer *et al.* hypothesised that induction of an autoimmune response against tumour associated self-antigens could be elicited by T cell homeostatic proliferation (Dummer et al., 2002). Following transfer of autologous or syngeneic T cells into mice lymphodepleted by sublethal irradiation, there was inhibition of tumour growth following challenge with melanoma or colon carcinoma. This depended on homeostatic expansion of transferred T cells in peripheral lymph nodes as the effect was reduced in the absence of lymph nodes (lymphotoxin-α gene knockout recipient mice) or if transferred cells lacked the ability to home to lymph nodes due to lack of β7 integrin and CD62L.

Gattinoni *et al.* demonstrated the concept of cytokine sinks by showing lymphodepletion enhanced adoptively transferred CD8 T cell anti-tumour efficacy, even in the genetic absence of endogenous regulatory T cells (Gattinoni, 2005). The enhanced efficacy following lymphodepletion was lost in recipient mice congenitally deficient in IL-15 and IL-7. They also demonstrated enhanced tumour control when recipient NK cells were removed by administration of *in vivo* depleting antibody. It is postulated that NK cells are one of the lymphoid cell populations that compete with transferred T cells for limiting cytokines. The role of NK cells as cytokine sinks was also shown with experiments comparing $Rag1^{-/-}$ to $Rag2^{-/-}\gamma_c^{-/-}$. $Rag1^{-/-}$ lack B and T lymphocytes but retain NK cells, whereas $Rag2^{-/-}\gamma_c^{-/-}$ lack NK cells as well as B and T cells. More extensive tumour regression was seen in $Rag1^{-/-}$ mice that were irradiated prior to adoptive cell transfer compared to non-irradiated recipients. In $Rag2^{-/-}\gamma_c^{-/-}$ recipients, there was no detectable difference between irradiated and non-irradiated mice, however adoptive cellular therapy was very efficacious in all $Rag2^{-/-}\gamma_c^{-/-}$ recipients. IL-15 is crucial for NK cell survival and proliferation *in vivo*, making NK cells likely candidates as cytokine sinks.

1.1.6.3 Lymphodepletion – Competition for Antigen Presenting Cells (APC)

In addition to competition with endogenous cells for limiting cytokines, adoptively transferred cells may also need to compete for antigen on the surface of antigen-presenting cells. Kedl *et al.* first demonstrated that T cells compete for access to antigen-bearing APCs (Kedl et al., 2000). They compared responses to the dominant ovalbumin epitope SIINFEKL and subdominant epitope KRVVFDKL in H-2^b mice. Transferred OT1 cells, transgenic for a high affinity SIINFEKL specific TCR, completely inhibited host ovalbumin specific T cell responses. OT1 cells also inhibited responses to unrelated peptide, providing it was presented on the same dendritic cells.

Lymphocyte activation, in the absence of lymphodepleting chemo-radiotherapy, will also be inefficient due to the absence of co-stimulatory molecules on immature dendritic cells. Lymphodepleting irradiation can reduce the absolute number of APCs *in vivo*, but also results in their activation. Within 6 hours of irradiation, C57Bl/6 spleens contain activated CD11c+ DC aggregated in the T cell areas (Zhang et al., 2002). Irradiation has also been shown to increase the frequency of APC producing the inflammatory cytokine TNFα (Brown et al., 2004, Hill et al., 1997), to upregulate surface expression of CD86 and I-Ab (MHC class II molecule) and DCs to release substantially more IL-12 (Zhang et al., 2002). Potential mechanisms for activation of APC include; translocation of Toll-like receptor agonists into the blood after mucosal damage (Hill et al., 1997) and increased levels of pro-inflammatory cytokines such as TNF (Sherman et al., 1991), IL-1 (Xun et al., 1994) and IL-4 (Rigby et al., 2003).

Systemic chemotherapy or irradiation prior to adoptive immunotherapy for malignant disease can result in apoptosis or necrosis of tumour cells. Russo *et*

al. showed that when attempting transduction of dendritic cells (DC) with a retroviral vector coding a cytoplasmic tumour antigen, there was dissociation between integration of vector DNA and the ability to stimulate a tumour-antigen specific response (Russo et al., 2000). DCs cultured with irradiated MAGE-3-expressing cells could stimulate a response against this tumour antigen in T cells obtained from a patient with MAGE-3 positive melanoma.

1.2 Post transplant Immunosuppressive Regimens

To use adoptive immunotherapy in the post transplant setting it is important to understand the actions of immunosuppressive medication used in these patients. The ability to transplant solid organs and haematopoietic stem cells has reduced mortality and morbidity for a number of conditions. It is the discovery and refinement of immunosuppressive medication that has enabled transplantation to become a standard treatment. Following SOT, the aim of immune suppression is to reduce the risk of the transplanted organ being recognised and rejected by the recipient's immune system. Following HSCT, the aim of immunosuppression is to reduce the graft recognising the recipient as foreign and causing graft versus host disease as well as preventing graft rejection by host immune cells recovering after conditioning.

Immunosuppressive regimes vary depending on multiple factors including the organ transplanted, the degree of HLA-match, the toxicity of specific immunosuppressive agents as well as the consequences of rejection e.g. immunosuppression following a heart transplant is greater than following a renal transplant. In HSCT, conditioning therapy and immunosuppression will depend on the disease for which the transplant is being performed, patient age and co-morbidities, the stem cell source, risk of GvHD and viral reactivation. Examples of immunosuppressive agents in common use are shown in table 1.

Here I briefly discuss some of the most commonly used drugs, their mechanism of action, side effects and use in post-transplant immunosuppression.

Table 1.1 Drugs used in Post-Transplant Immunosuppression

Class of drug		Example
Glucocorticoids		Prednisolone
Small molecules	Calcineurin inhibitors	Ciclosporin Tacrolimus
	Mammalian Target Of Rapamycin inhibitors	Sirolimus
	Purine synthesis inhibitors	MMF
	Pyrimidine synthesis inhibitors	Azathioprine
Biological agents	Depleting antibodies	ATG (polyclonal) Alemtuzumab (CD52) Rituximab (CD20) Muromonab (CD3)
	Non-depleting antibodies and fusion proteins	Daclizumab (CD25) Basiliximab (CD25) Belatacept (CTLA-4)
C5 Inhibitor		Eculizumab
Protease Inhibitor		Bortezomib

1.2.1 Corticosteroids

Corticosteroids are a mainstay of immunosuppressive regimen both in induction and maintenance as well as in acute rejection and GvHD. They are agonist of the glucocorticoid receptor and prevent production of cytokines (including IL-1, IL-2 and TNF-α) and vasoactive substances (e.g. prostaglandins). Steroids have many unwanted side-effects and newer regimens in SOT have been designed to reduce or completely remove their usage (Guerra et al., 2011, Chhabra et al., 2012, Rajab et al., 2006, Oaks et al., 2001, Xing et al., 2013). The side effects include glucose intolerance, hypertension, cataracts, psychiatric disturbance, osteoporosis, avascular necrosis and development of cushingoid features (Veenstra et al., 1999, Stanbury and Graham, 1998).

1.2.2 Calcineurin inhibition – Ciclosporin

Ciclosporin has been a mainstay of transplantation for over 40 years and has been utilised in both induction and maintenance of immunosuppression. It is an 11 amino acid cyclic peptide produced by the fungus *Tolypocladium inflatum (Borel et al., 1976).* It binds to cyclophilin and subsequently calcineurin, abolishing its dephosphorylation activity. Inhibition of calcineurin prevents IL-2 production and T-cell activation. Close monitoring of trough levels is necessary with adjustment in dosing. The main side effects of nephrotoxicity and hypertension have resulted in many investigators trialling regimes without ciclosporin or with reduced dosing (Ekberg et al., 2007). In renal transplantation a randomised trial comparing the three most common immunosuppressive regimen (ciclosporin and sirolimus, MMF and tacrolimus, tacrolimus and sirolimus) showed the combination of MMF and tacrolimus to be most favourable, with lower levels of acute rejection and better graft function (Guerra

et al., 2011). Following heart transplant reducing the use of ciclosporin, particularly during maintenance immunosuppression, has also been shown to be advantageous (Aleksic et al., 2000, Baryalei et al., 2003, Zuckermann et al., 2001, Hamour et al., 2007) however there are data to contradict this (Hunt et al., 2007).

1.2.3 MTOR Inhibition – Sirolimus

Sirolimus, also known as rapamycin, binds to FK-binding protein 12 which inhibits the mammalian target of rapamycin complex 1, resulting in inhibition of signal 3 by stopping RNA translation and preventing G_1-S phase progression. It also inhibits IL-2 and IL-4 dependent proliferation of lymphocytes. It causes minimal nephrotoxicity, which makes it an attractive option in patients with impaired renal function. It can cause impaired wound healing and thrombocytopenia. Potentially severe lung toxicity precludes its use following lung transplantation.

1.2.4 Purine synthesis inhibition – MMF

MMF inhibits production of guanine nucleotides. It significantly reduces renal transplant loss (Ojo et al., 2000) and its use has generally replaced azathioprine, which was used in the first renal transplants. This was due to limited toxicity and a reduction in acute rejection rates in renal transplant recipients (Knight et al., 2009). The actions and use of MMF are described in section 1.4 (pp. 51).

1.2.5 Pyrimidine synthesis inhibition – Azathioprine

Azathioprine, in combination with glucocorticoid, was the first immunosuppressive agent used in transplantation (Murray et al., 1963). It is a prodrug that is converted to 6-Mercaptopurine (6-MP) by non-enzymatic

cleavage of a thioether group. 6-MP undergoes further metabolism to form several metabolites. The main active metabolite, methyl-thioinosine monophosphate is a pyrimidine synthesis inhibitor that blocks the enzyme amidophosphoribosyltransferase. Another metabolite, thioguanosine triphosphate is incorporated into and interferes with RNA. A further metabolite, thio-deguanosine triphosphate, is incorporated into DNA, impeding synthesis.

The main adverse effect of azathioprine is myelosuppression, which is a potentially lethal complication. The degree of suppression is related to dose and is most marked in people with a genetic deficiency of thiopurine S-methyltransferase. Cessation of drug can result in recovery of marrow function, however to avoid this side effect, azathioprine has generally been replaced by ciclopsorin and MMF. Ciclosporin results in longer survival times when compared to azathioprine in heart transplantation (Modry et al., 1985). MMF has been shown to cause less bone marrow suppression, fewer infections and less acute rejection. Despite this many studies suggest little overall benefit of MMF compared to azathioprine and it remains cheaper than MMF.

1.2.6 Biological agents – Antithymocyte globulin (ATG)

Polyclonal antithymocyte globulins, raised in rabbits or horses, have been used in induction immunosuppressive regimen since the 1970s. They result in the depletion of thymocytes in the recipient. In general, ATG is being replaced by newer monoclonal antibodies in SOT regimen (Hao et al., 2012) particularly those with a low risk of rejection, but its use continues in HSCT regimen (Bacigalupo, 2005). During infusion the xenogenic proteins can cause allergic reactions including anaphylaxis. There is an associated increased risk of CMV infection and post-transplant malignancy.

1.2.7 Biological agents – Alemtuzumab

Alemtuzumab is a humanized monoclonal anti-CD52 antibody that is lymphocyte-depleting. It is used in HSCT reduced intensity conditioning regimes where it can deplete both recipient and donor lymphocytes, reducing GvHD and treatment related mortality (Poire and van Besien, 2011). Its use is increasing in SOT, where it has been shown to reduce the incidence of acute rejection compared to conventional therapy (Hanaway et al., 2011).

1.2.8 Biological agents – Basiliximab and Daclizumab

Anti-CD25 antibodies (Basiliximab and Daclizumab) are chimeric humanized monoclonal antibodies. CD25 is the IL-2 receptor α chain found on activated T cells. Ligation inhibits T cell activation and can result in depletion. They are well tolerated and are established as first line treatment for induction of immunosuppression for SOT. While they have been shown to reduce acute rejection episodes, there is no increase in kidney or patient survival. Their use may enable elimination or reduction of more toxic immunosuppressive agents (Salis et al., 2008).

1.2.9 Biological agents – Rituximab

Rituximab is a monoclonal anti-CD20 molecule that depletes B cells. It is not used as a routine part of post-transplant immunosuppressive regimen but can be added pre transplant in patients with preformed alloantibodies (Vo et al., 2010). It has also been used for the treatment of antibody-mediated rejection, however data from larger studies is needed to prove efficacy (Becker et al., 2004, Roberts et al., 2012).

1.2.10 Use of immunosuppressive agents in combination

Since the first SOT, combinations of different immunosuppressive drugs rather than single agents have been given to prevent rejection. Different drugs are used in the induction/conditioning phase, immediately prior to and after transplantation, compared to long-term maintenance immunosuppression. In renal transplantation, international guidelines recommend combination therapy including a biologic agent (CD25-antibody or ATG) at induction, followed by glucocorticoids, calcineurin inhibitor and anti-proliferative agent (Kasiske et al., 2010). The recommended calcineurin inhibitor is tacrolimus and MMF is the suggested anti-proliferative agent. There is significant variability in practice and, as discussed previously, attempts to reduce toxicity by reduction or elimination of glucocorticoids and ciclosporin from the immunosuppressive regime.

Similarly for heart transplantation, a combination regimen of calcineurin inhibitor (tacrolimus) and anti-proliferative agent (MMF) or MTOR inhibitor are recommended with biological agent induction especially to enable more rapid reduction in other agents (Costanzo et al., 2010).

In HSCT, many chemo-radiotherapy induction regimes contain a lymphocyte-depleting biological agent such as alemtuzumab or ATG. Other techniques, such as immunomagnetic selection of CD34 positive stem cells, have been used to deplete T-cells from the graft prior to transfer to the recipient. A survey of European transplant centres, showed wide variability of agents used for prophylaxis. Ciclosporin and MMF were the two most commonly used drugs for GvHD prophylaxis (Ruutu et al., 2012).

1.3 Effect of pharmacological immunosuppression on cellular therapy

The discovery and development of immunosuppressive medication has been instrumental in the treatment of autoimmune disease and has allowed millions of patients worldwide to receive HSCT and SOT from both related and unrelated donors. Despite the clear benefits, suppressing the immune system also results in many side effects. The individual characteristics of each drug determine the side effect profile but increased rates of infection and malignancy relate to the unavoidable reduction in immunosurveillance by suppression of lymphocytes.

Post-SOT adoptive immunotherapy for EBV and CMV has not been as successful as that post-HSCT. Two possible explanations are:

1) Pre-transplant lymphodepletion - Conditioning for HSCT causes lymphodepletion providing an immunological niche for transferred cells to expand into with reduced competition from other lymphocytes. EBV-CTLs administered post-SOT enter a lymphocyte replete patient and therefore more competition for cytokines.

2) Ongoing immunosuppression – patients receiving EBV-CTLs post-HSCT had ceased immunosuppression, while post-SOT immunosuppression was usually continued.

Although CMV-specific CD8+ T cells can be detected at normal levels in patients receiving immunosuppressive therapy, these cells display impaired functionality as shown by reduced IFN-γ secretion (Engstrand et al., 2003).

This supports the hypothesis that immunosuppression will inhibit transferred cells.

Immunosuppression is also able to impair adoptive immunotherapy with CD4+ T cells. A study investigating the effect of standard immunosuppressants (Ciclosporin, MMF, glucocorticoids and rapamycin) on Anti-Aspergillus T_H1 cells showed that all doses of ciclosporin, MMF and glucocorticoids used significantly decreased the number of viable anti-aspergillus cells in culture (Tramsen et al., 2014). The doses reflected target doses in serum and doses above and below this, however it is uncertain whether this effect will also occur *in vivo*.

If transferred cells were conferred with resistance to immunosuppressants, it is hypothesised that they would improve the outcome of adoptive immunotherapy post-SOT and allow immunosuppression to continue post-HSCT. Two groups have published strategies to engineer cells with resistance to calcineurin inhibitors prior to adoptive transfer. Brewin *et al.* (Brewin et al., 2009) transduced cells with calcineurin mutants which conferred resistance to tacrolimus and/or ciclosporin and demonstrated *in vitro* efficacy of the resistance gene. Continuation of this work was presented recently showing efficacy in a xenogenic mouse model (Ricciardelli et al., 2014). De Angelis *et al* (De Angelis et al., 2009) knocked down a binding protein (FKBP12) on which tacrolimus depends using a specific small interfering RNA and demonstrated activity in a xenogenic mouse model of PTLD during tacrolimus therapy.

As outlined in section 1.2, many other immunosuppressants are in common usage either as monotherapy or in combination. Strategies to provide resistance to additional immunosuppressants would expand the potential for

adoptive immunotherapy post transplant and during therapy for autoimmune disease. Several teams have investigated engineering resistance to mycophenolate mofetil (MMF). A mutant form of the enzyme targeted by the drug was identified from a murine cell line exposed to increasing concentrations of MMF (Hodges et al., 1989, Lightfoot and Snyder, 1994). Initial work focused on enabling selection of transduced cells (Yam et al., 2006, Sangiolo et al., 2007). During preparation of this manuscript, a further report using the same gene alongside a gene conferring methotrexate resistance combined in the same vector has also been published (Jonnalagadda et al., 2013). In this work, I look to assess the utility of this MMF resistance gene for adoptive immunotherapy during immunosuppressive therapy.

1.4 Mycophenolate Mofetil

The immunosuppressant mycophenolate mofetil (MMF) is routinely used in the prevention of allograft rejection (Matas et al., 2013) and treatment of autoimmune diseases (Appel et al., 2005, Richardson et al., 2000, Boehm and Bieber, 2001, Moore and Derry, 2006). Mycophenolic acid (MPA), the active metabolite of MMF, non-competitively inhibits Inosine-5'-monophosphate dehydrogenase (IMPDH).

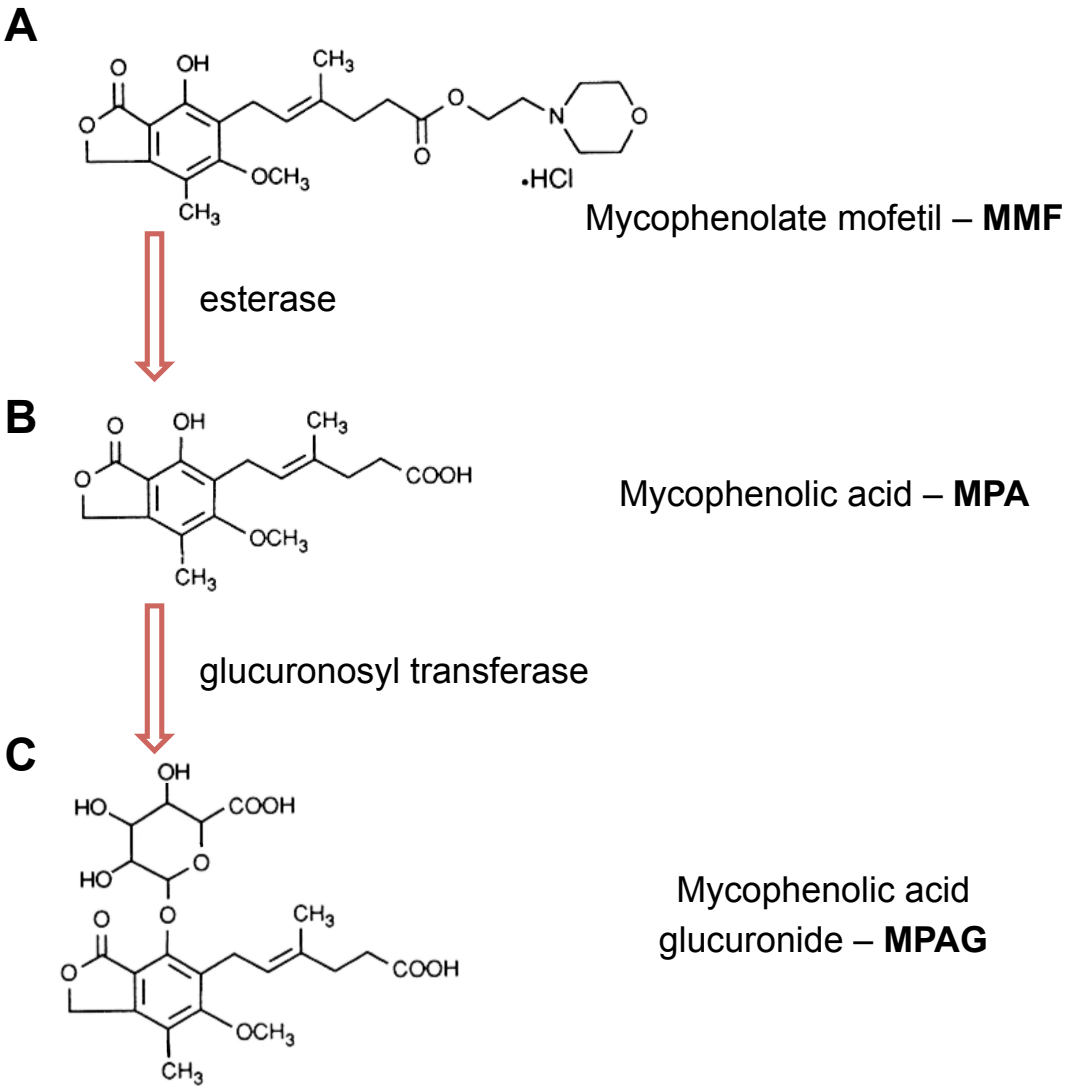
1.4.1 Identification and development of MPA/MMF

MPA was originally identified in 1896 as a fermentation product of *penicillium brevicompactum* (Gosio, 1896), having been isolated from spoiled corn during investigations into the cause of pellagra. Gosio showed that the acidic fermentation product he had isolated was able to inhibit the growth of anthrax bacillus. The same compound was 're-discovered' by Alsberg and Black in 1913, when they isolated it from deteriorated Italian corn and named the compound mycophenolic acid. They established the chemical formula $C_{17}H_{20}O_6$. The chemical structures of MMF, MPA and the inactive degradation product MPA glucuronide are shown in figure 1.2.

The action of MPA on IMPDH, and its ability to inhibit nucleic acid synthesis in eukaryotic cells was established in 1969 (Franklin and Cook, 1969). The lethal dose for an adult mouse was determined to be ~10mg when administered intravenously (~500µg/g) and 40mg orally (Florey et al., 1946). Initial investigations showed that MPA had antimicrobial, antineoplastic and immunosuppressive activity *in vitro*.

Figure 1.2 Chemical structure of mycophenolate mofetil, mycophenolic acid and mycophenolic acid glucuronide

The chemical structure of MMF (2-morpholinoethyl (E)-6-(1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)-4-methyl-4-hexenoate) is shown (A). MMF has a molecular formula of $C_{23}H_{31}NO_7$ and a molecular weight of 433.5 Daltons. Following intravenous or oral administration, MMF is rapidly hydrolysed to MPA by serum and tissue esterases. MPA has the molecular formula $C_{17}H_{20}O_6$ and its chemical structure is shown (B). MPA is metabolised in the liver by glucuronosyl transferase to form the inactive Mycophenolic acid glucuronide (C).



1.4.2 Purine metabolism

There are two major pathways of purine synthesis, *de novo* and salvage (Figure 1.3). In the *de novo* pathway, ribose-5-phosphate and adenosine triphosphate (ATP) are converted to 5-phosphoribosyl-1-pyrophosphate (PRPP) catalyzed by the enzyme PRPP synthetase. PRPP is then converted to inosine monophosphate (IMP), from which guanine or adenine nucleotides can be synthesized.

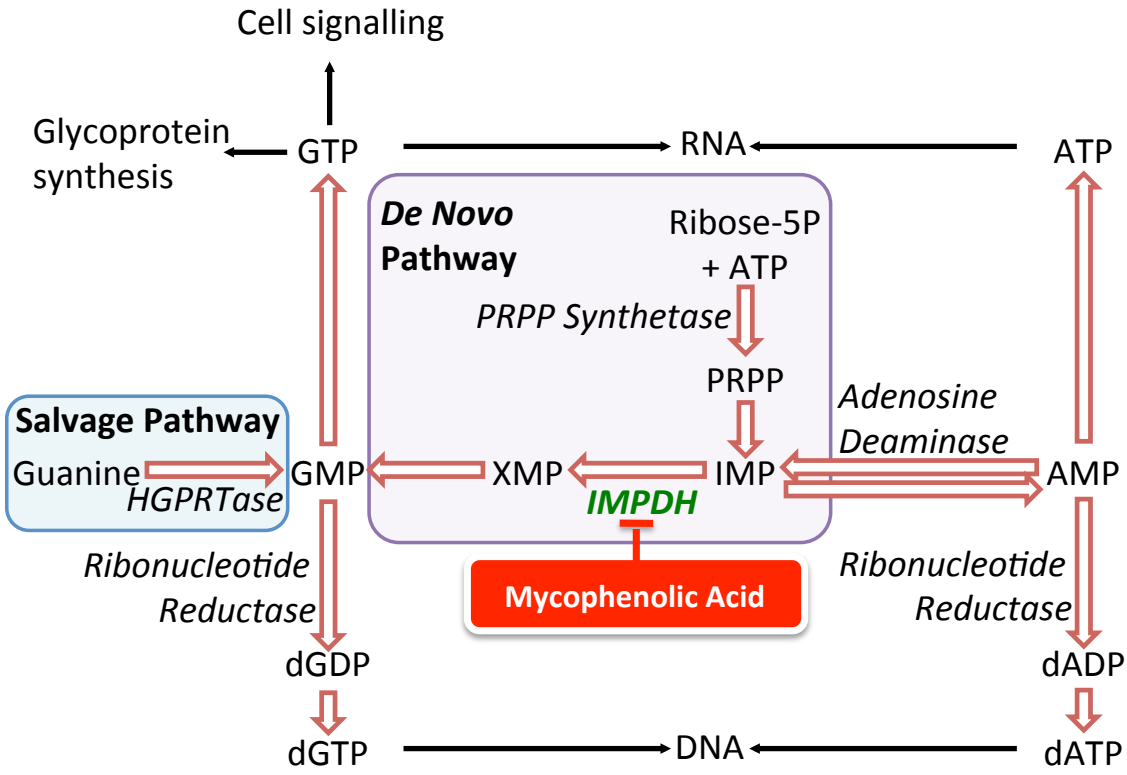
The conversion of IMP to XMP, catalyzed by IMPDH, is the first committed and rate-limiting step in guanine nucleotide biosynthesis. Guanosine monophosphate (GMP) is synthesized from XMP catalyzed by GMP synthetase. Guanine nucleotides serve as precursors for RNA and DNA, the energy source for translation, the co-factor for G-proteins, precursors for glycosylation, the precursor for tetrahydrobiopterin synthesis as well as important allosteric regulators and signaling molecules (Allison and Eugui, 2000).

To generate adenosine monophosphate (AMP), IMP undergoes a two-step conversion, catalyzed by adenylosuccinate synthetase and adenylosuccinate lyase respectively. Generation of dGTP and dATP involves reactions catalysed by ribonucleotide reductase.

The guanine salvage pathway involves the formation of GMP from guanine and PRPP, a reaction catalyzed by hypoxanthine-guanine phosphoribosyltransferase (HGPRT).

Figure 1.3 Purine metabolism

The two major pathways of purine synthesis, *de novo* and salvage are shown. In the de novo pathway, 5-phosphoribosyl- 1-pyrophosphate (PRPP) is formed from ribose-5-phosphate and adenosine triphosphate (ATP) by PRPP synthetase. PRPP is then converted to inosine monophosphate (IMP). Adenosine monophosphate (AMP) is synthesized from IMP in two steps, catalyzed by adenylosuccinate synthetase and adenylosuccinate lyase respectively. Guanosine monophosphate (GMP) is converted from IMP by the action of two enzymes, IMPDH and GMP synthetase. GMP is also generated via the salvage of guanine nucleotides catalyzed by hypoxanthine-guanine phosphoribosyltransferase (HGPRT). Adenosine diphosphate (ADP) and guanosine diphosphate (GDP) are converted to deoxyadenosine diphosphate (dADP) and deoxyguanosine diphosphate (dGDP) respectively by ribonucleotide reductase.



Feedback within the purine synthesis pathway occurs via two routes. When adenosine nucleotides exceed guanosine nucleotides, PRPP synthetase is inhibited (Garcia et al., 1977). Excess dATP, compared to dGTP, causes inhibition of ribonucleotide reductase (Kashlan et al., 2002).

1.4.2.1 Inherited defects of purine metabolism

Inherited deficiency of adenosine deaminase (*de novo* pathway) causes severe immunodeficiency (Giblett et al., 1972), but Lesch-Nyhan syndrome caused by HGPRT deficiency (salvage pathway) results in severe neurological symptoms but no immunodeficiency (Seegmiller et al., 1967). This suggested that human lymphocytes are relatively dependent on the *de novo* pathway of purine synthesis whereas the salvage pathway catalyzed by HGPRT is not essential.

1.4.3 Inosine-5'-Monophosphate Dehydrogenase (IMPDH)

With the exception of protozoan parasites such as Giardia lamblia (Morrison et al., 2007) and Trichomonas vaginalis (Carlton et al., 2007), the IMPDH pathway appears to be present in every organism. The human and murine sequences differ by only six amino acids (figure 1.4). Moreover, many organisms contain multiple genes encoding IMPDH. Two distinct cDNAs of human IMPDH have been cloned, producing two isoforms, designated types 1 and 2 (Collart and Huberman, 1988, Natsumeda et al., 1990). The genes are encoded on chromosomes 7 and 3 respectively. Both isoforms contain 514 amino acids, of which 84% are identical (Natsumeda et al., 1990), and are almost indistinguishable in their kinetic properties.

Figure 1.4 Amino acid sequences of murine and human Inosine-5'-Monophosphate Dehydrogenase (IMPDH2)

Green highlights the six differences between the amino acid sequence of murine top and human bottom IMPDH2

```
muIMPDH2    1 MADYLISGGTSYVPDDGLTAQQLFNCGDGLTYNDFLILPGYIDFTADQVDLTSALTKKIT
huIMPDH2    1 MADYLISGGTSYVPDDGLTAQQLFNCGDGLTYNDFLILPGYIDFTADQVDLTSALTKKIT

muIMPDH2   61 LKTPLVSSPMDTVTEAGMAIAMALTGGIGFIHHNCTPEFQANEVRKVKKYEQGFITDPVV
huIMPDH2   61 LKTPLVSSPMDTVTEAGMAIAMALTGGIGFIHHNCTPEFQANEVRKVKKYEQGFITDPVV

muIMPDH2  121 LSPKDRVRDVFEAKARHGFCGIPITDTGRMGSRLVGIISSRDIDFLKEEEHDCFLEEIMT
huIMPDH2  121 LSPKDRVRDVFEAKARHGFCGIPITDTGRMGSRLVGIISSRDIDFLKEEEHDRFLEEIMT

muIMPDH2  181 KREDLVVAPAGTTLKEANEILQRSKKGKLPIVNEDDELVAIIARTDLKKNRDYPLASKDA
huIMPDH2  181 KREDLVVAPAGVTLKEANEILQRSKKGKLPIVNENDELVAIIARTDLKKNRDYPLASKDA

muIMPDH2  241 KKQLLCGAAIGTHEDDKYRLDLLAQAGVDVVVLDSSQGNSIFQINMIKYIKDKYPNLQVI
huIMPDH2  241 KKQLLCGAAIGTHEDDKYRLDLLALAGVDVVVLDSSQGNSIFQINMIKYIKEKYPSLQVI

muIMPDH2  301 GGNVVTAAQAKNLIDAGVDALRVGMGSGSICITQEVLACGRPQATAVYKVSEYARRFGVP
huIMPDH2  301 GGNVVTAAQAKNLIDAGVDALRVGMGSGSICITQEVLACGRPQATAVYKVSEYARRFGVP

muIMPDH2  361 VIADGGIQNVGHIAKALALGASTVMMGSLLAATTEAPGEYFFSDGIRLKKYRGMGSLDAM
huIMPDH2  361 VIADGGIQNVGHIAKALALGASTVMMGSLLAATTEAPGEYFFSDGIRLKKYRGMGSLDAM

muIMPDH2  421 DKHLSSQNRYFSEADKIKVAQGVSGAVQDKGSIHKFVPYLIAGIQHSCQDIGAKSLTQVR
huIMPDH2  421 DKHLSSQNRYFSEADKIKVAQGVSGAVQDKGSIHKFVPYLIAGIQHSCQDIGAKSLTQVR

muIMPDH2  481 AMMYSGELKFEKRTSSAQVEGGVHSLHSYEKRLF
huIMPDH1  481 AMMYSGELKFEKRTSSAQVEGGVHSLHSYEKRLF
```

Type I, the preponderant isoform in the retina, spleen and resting peripheral blood mononuclear cells, is constitutively expressed and is not inducible (Nagai et al., 1991). Type 2 is selectively up-regulated in neoplastic (Natsumeda et al., 1990) and replicating cells and emerges as the dominant species (Nagai et al., 1991, Nagai et al., 1992). Most tissues express both isozymes to varying extents (Jain et al., 2004, Senda and Natsumeda, 1994, Bowne et al., 2006a). IMPDH1 knockout mice display mild retinopathy, however IMPDH2 null mice die during embryogenesis. A recent report shows that the isoforms display different nucleotide binding with differential regulation by a mechanism involving binding to the subdomain by AMP (IMPDH2) and ATP (IMPDH1) (Thomas et al., 2012).

The type 2 isoform was found to be 4.8 times more sensitive to MPA than the type 1 isoform (Carr et al., 1993). The fact that the type 2 isoform of IMPDH is predominantly expressed in activated lymphocytes may partly explain why MPA is relatively selective for lymphocytes.

1.4.3.1 IMPDH structure

IMPDH proteins form stable tetramers with square planar geometry that may contain both isoforms. The x-ray crystal structure identifies monomers containing two domains: the core catalytic domain, which is a (α/β)8 barrel, and the flanking subdomain containing two CBS domains (Carr et al., 1993)'(Nimmesgern et al., 1999, Gan et al., 2002).

CBS domains are conserved sequences found in a wide range of proteins in all species. The secondary structure, $\beta_1\alpha_1\beta_2\beta_3\alpha_2$, folds into a globular structure with three antiparallel β sheets together and the two α-helices on the other side. CBS domains are always found in pairs that tightly associate via their β-

sheets in what is termed a Bateman domain. ATP binds to the Bateman domains of tetrameric IMPDH in a positive, cooperative way (Cornuel et al., 2002, McLean et al., 2004, Mortimer and Hedstrom, 2005, Bowne et al., 2006b). ATP binding activates IMPDH catalytic activity (Scott et al., 2004). ATP binding and activation is abolished by a single amino acid mutation in the second CBS domain (R224P) which when present in IMPDH 1 is a cause of autosomal dominant retinitis pigmentosa. In addition to activation of the enzymatic domain, the CBS subdomains have also been shown to:

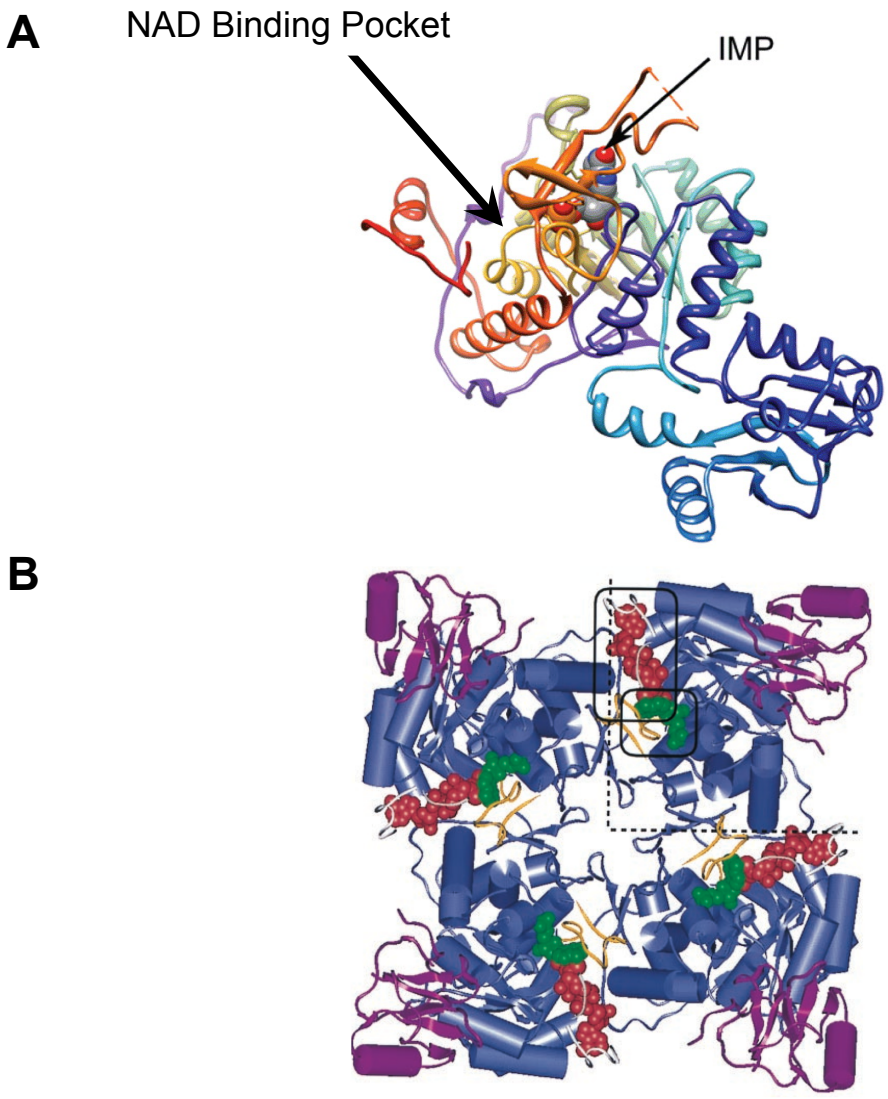
1) regulate nucleotide pools (Pimkin and Markham, 2008).
2) associate with polyribosomes, suggesting involvement in the regulation of translation (Mortimer et al., 2008).

The junction between the catalytic domain and the subdomain is flexible and the relative orientation can vary by as much as 120 degrees in different crystal structures (Figure 1.5) (Colby et al., 1999).

The enzymatically active site of the catalytic domain is located in the loops on the C-terminal ends of the β sheets. The catalytic loop, the C-terminal segment and a flap segment display structural mobility. This is critical for enzymatic activity, with a different conformation generated for each step of the catalytic cycle. A large segment between β8 and α8 forms a flap, the distal portion of which moves in and out of the active site during the catalytic cycle; the open conformation is required for the dehydrogenase reaction while the closed conformation is used in the hydrolysis step (Hedstrom and Gan, 2006). Both the key catalytic residue Cys319 and most of the residues that interact with IMP are conserved across species. However, the NAD site and the flap are highly divergent.

Figure 1.5 Structure of Inosine-5'-Monophosphate Dehydrogenase (IMPDH)

(A) An IMPDH2 monomer is shown as a ribbon diagram with IMP bound in the enzymatic domain. (B) Human Type II IMPDH tetramer with bound dinucleotide analogue (circled, red) and substrate analogue (circled, green). The dinucleotide binds at the monomer–monomer interface (dotted lines). The following structures are illustrated: catalytic β-barrel domain (blue), flanking domain (magenta), active site loop (yellow) and active site flap fragments (white). IMP – Inosine monophosphate, NAD – Nicotinamide adenine dinucleotide



From Colby et al. Crystal structure of human type II inosine monophosphate dehydrogenase: implications for ligand binding and drug design
PNAS 1999;96 (7) 3531-3536

1.4.3.2 Enzymatic action of IMPDH

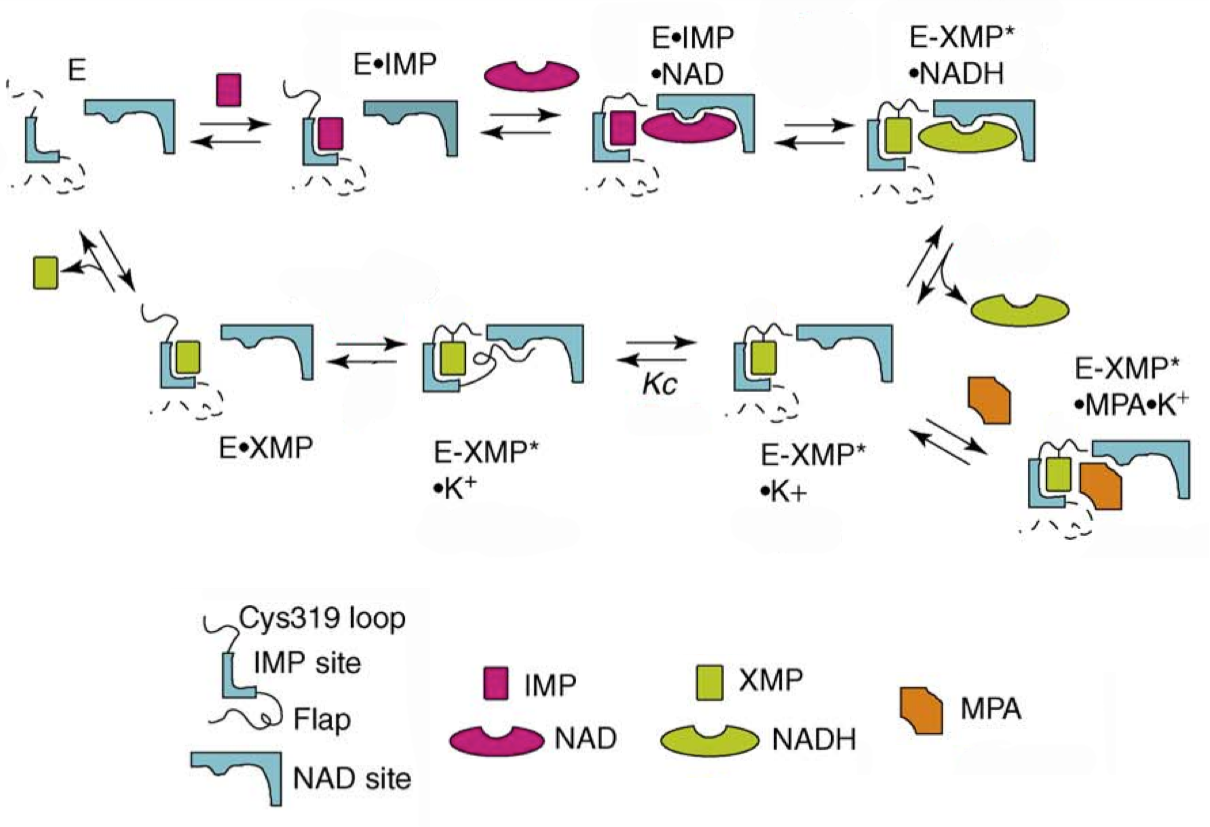
IMPDH catalyzes the nicotinamide adenine dinucleotide (NAD)-dependent oxidation of IMP to xanthosine monophosphate (XMP), which is the committed step in the *de novo* biosynthesis of guanine nucleotides (Crabtree and Henderson, 1971, Jackson et al., 1975). The reaction is irreversible and was initially thought to follow bi-bi sequential ordered kinetics (Carr et al., 1993); however, more recently a more random mechanism has been shown to occur (Heyde et al., 1976, Heyde and Morrison, 1976). The mechanism of action of IMPDH is shown diagrammatically in Figure 1.6. IMPDH catalyzes two chemical transformations: firstly following IMP binding a dehydrogenase reaction to form NADH and the covalent intermediate E-XMP* and then a hydrolysis reaction which converts E-XMP* into XMP.

MPA and the nicotinamide portion of NAD^+ have similar volumes and electronic properties(Makara et al., 1996). Mycophenolic acid (MPA) traps E-XMP* within IMPDH, the crystal structure of the E-XMP*•MPA complex has been solved (Sintchak et al., 1996, Fleming et al., 1996, Link and Straub, 1996) which shows that MPA stacks against the purine ring in a similar manner to the nicotinamide ring of NAD^+ (Sintchak et al., 1996). The strong preference for E-XMP* makes MPA an uncompetitive inhibitor with respect to both IMP and NAD^+.

Figure 1.6 The kinetic mechanism and conformational transitions of the Inosine-5'-Monophosphate Dehydrogenase (IMPDH) reaction.

The pathway where IMP binds first is depicted for clarity. The Cys319 loop and the flap are initially disordered (denoted by the dashed lines). When IMP binds, the loop becomes more ordered. NAD+ binds and the dehydrogenase reaction occurs rapidly to produce E–XMP*•NADH. The release of NADH is slower than hydride transfer. MPA binds to the vacant NADH site of E–XMP*. Alternatively, the flap folds into the NADH site, positioning Arg418 to activate water. The hydrolysis of E–XMP* is rate-limiting, and is believed to trigger the release of K+ and the opening of the flap and loop.

IMP - Inosine monophosphate; MPA – Mycophenolic acid; NAD - Nicotinamide adenine dinucleotide; XMP – Xanthine monophosphate



Adapted from L Hedstrom and Lu Gan IMP dehydrogenase: structural schizophrenia and an unusual base
Current Opinion in Chemical Biology 2006, 10:520–525

1.4.3.3 Other functions of IMPDH

In addition to the enzymatic function of IMPDH, several other functions have been proposed. Kozhevnikova *et al.* showed that in Drosophila IMPDH not only controls cell proliferation by controlling the guanine nucleotide pool but is also a DNA-binding transcriptional repressor, binding to CT-rich DNA elements (Kozhevnikova et al., 2012). DNA binding by IMPDH reduces cell proliferation by reducing histone gene and E2f expression. DNA binding is through the CBS domain.

IMPDH2 has been shown to rapidly recruit to lipid raft after Toll-like receptor 2 (TLR2) activation. It causes increased phosphatase activity of SHP1 resulting in inactivation of PI3K and thereby negatively regulation TLR2 signalling (Toubiana et al., 2011).

IMPDH is a rate-determining factor in the regulation of proliferation by p53 (Liu et al., 1998). Constitutive IMPDH expression prevents growth suppression while inhibition of IMPDH mimics over-expression of p53.

1.4.3.4 Mutations in IMPDH

As stated in section 1.4.3.1, the R224P mutation of IMPDH1 causes autosomal dominant retinitis pigmentosa as does D226N and V268I (Mortimer and Hedstrom, 2005). Additionally two mutations have been linked to Leber congenital amaurosis, R105W and N198K. While IMPDH2 has low genetic diversity, one variant (L263F) displays marked reduction in enzymatic activity (Wang et al., 2007).

Within this work I have used a mutant form of IMPDH generated by incubation of murine neuroblastoma cells in increasing concentrations of MPA (Hodges et al., 1989). The resulting cells were 10,000 fold more resistant to MPA induced

growth inhibition. The cells had increased expression of IMPDH due to increased copy number resulting in 10-20 fold increase in IMPDH mRNA and IMPDH accounted for 20% of soluble proteins. MPA resistance was also caused by mutation in the IMPDH gene. 4 nucleotide changes were identified, one of which was present in the parental tumour cell line and another did not result in a codon change. The remaining 2 mutations caused 2 amino acid substitutions, T333I and S351Y, that result in 2400-fold increased resistance to MPA (Lightfoot and Snyder, 1994). The mutations also cause a reduction in enzymatic activity, with the estimated K_{cat} 10% of wild-type enzyme.

Mutational analysis of the IMPDH catalytic domain has identified mutants with reduced enzymatic activity (Futer et al., 2002). I have used a mutant with unrecordable catalytic activity, C331A, which is designated $IMPDH2^{CS}$ and the wild-type gene $IMPDH2^{WT}$.

1.4.4 Pharmacokinetics of MMF

MMF is rapidly absorbed following oral administration. After oral or intravenous administration, MMF is hydrolysed to pharmacologically active MPA by serum and tissue esterases. Following oral administration, MMF itself is not measurable in transplant patients or animals (Lee et al., 1990). Up to 98% of MPA binds to albumin, with only the free portion being active (Nowak and Shaw, 1995). MPA is metabolised in the liver by glucuronosyl transferase to form the inactive mycophenolic acid glucuronide (MPAG) (Sweeney et al., 1972). MPAG is primarily eliminated in the urine. MPA also exhibits enterohepatic recirculation where inactive MPAG is excreted in bile (Shaw and Nowak, 1995). This is metabolised by intestinal bacteria to reform MPA and both MPA and MPAG are reabsorbed in the gut (Bullingham et al., 1998).

Peak MPA levels typically occur between 20 and 40 minutes after administration but there is significant inter- and intra-patient variability in pharmacokinetics. The half-life of MPA is approximately 18 hours and enterohepatic recirculation can result in a small secondary peak in MPA levels approximately 16 hours post administration. Dosing schedules require administration of MMF two or three times daily. MPA levels are usually assessed pre-dose (trough). The therapeutic range for pre-dose levels, calculated from reported successful immunosuppression, is given as 1-3.5mg/l, equating to a concentration of 3.1-10.9μM.

1.4.4.1 Clinical monitoring of MPA levels

A 2008 review for the US Department of Health concluded that routine monitoring of MPA levels was not recommended (Oremus et al., 2008). There is however evidence that monitoring MPA levels and appropriate adjustment of doses may improve outcomes (Neumann et al., 2008, Le Meur et al., 2007, Hiwarkar et al., 2011, Tredger et al., 2004, Borrows et al., 2006). Monitoring of MPA is usually performed by high performance liquid chromatography, an EMIT immunoassay is also available but this reacts with both MPA and MPAG which further complicates interpretation of results (Weber et al., 2002).

1.4.5 Antimicrobial activity of MPA

Following the discovery of penicillin, many fungal metabolites were investigated for their antibiotic properties. Rapid proliferation is a characteristic of microbial infections, so IMPDH is an attractive antimicrobial target. Antimicrobial utility may be limited by salvage of xanthine in addition to guanine and/or guanosine by many pathogens. Xanthine salvage enables several bacteria to retain normal virulence even in the absence of IMPDH (Ivanovics et al., 1968, McFarland and Stocker, 1987, Noriega et al., 1996, Straley and Harmon, 1984). Inhibition of IMPDH has been shown to inhibit the growth of Staphylococcus aureus (Abraham, 1945, Harris, 1943), Plasmodium falciparum (Webster and Whaun, 1982), Eimeria tenella (Hupe et al., 1986), Tritrichomonas foetus (Hedstrom et al., 1990), Leishmania donovani (Wilson et al., 1991), Trypanosoma brucei (Wilson et al., 1994) Candida albicans (Kohler et al., 1997) and Cryptosporidium parvum (Striepen et al., 2004). Drug resistance has been demonstrated *in vitro*, by both amplification (Wilson et al., 1991, Wilson et al., 1994, Lightfoot and Snyder, 1994) and drug resistance mutations (Kohler et al., 1997, Lightfoot and Snyder, 1994, Farazi et al., 1997) in the IMPDH gene.

Additionally, MPA was shown to have *in vitro* anti-fungal (Florey et al., 1946, Noto et al., 1969) and anti-viral (Florey et al., 1946, Williams et al., 1968, Cline et al., 1969, Neyts et al., 1998) activity. However, MMF is not licensed for use in the treatment of fungal or viral infection. During MMF treatment, patients are at risk of primary viral infections and viral reactivation.

1.4.6 Antineoplastic activity of MPA

Rapidly dividing cells have a high demand for guanine nucleotides. This requirement cannot be sustained by salvage pathways and explains the importance of IMPDH in cancer. Tumour cells have been shown to contain increased levels of IMPDH mRNA, protein and activity (Collart et al., 1992, Nagai et al., 1991).

Early *in vitro* studies identified the anti-neoplastic activity of MPA (Williams et al., 1968, Carter et al., 1969, Suzuki et al., 1969). Additionally, MMF's antiangiogenic activity (Chong et al., 2006, Domhan et al., 2008) is desirable in treating malignancy. MPA induces differentiation and/or apoptosis of several cancer cell lines, including breast (Bacus et al., 1990), prostate (Floryk and Huberman, 2006, Floryk et al., 2004), melanoma (Kiguchi et al., 1990), leukemia (Collart and Huberman, 1990) and neuroblastoma (Messina et al., 2004, Messina et al., 2005).

Due to its anti-neoplastic action *in vitro*, MPA was investigated both in murine tumour models and clinical trials as a cancer chemotherapeutic agent. MPA was found to have variable efficacy in *in vivo* murine models (Suzuki et al., 1969, Planterose, 1969) and results from early clinical trials showed no benefit from MPA (Brewin et al., 1972). The antineoplastic efficacy of MPA *in vivo* is limited by glucuronidation of phenolic oxygen, inactivating the drug (Franklin et al., 1996). Cancer cells appear to have a higher capacity for glucuronidation than normal cells.

1.4.7 Immunosuppressive activity of MPA

The first indication that MPA had immunosuppressive activity was seen in experiments performed by Planterose (Planterose, 1969). He used an *in vivo* tumour model triggered by mouse sarcoma virus and noted that splenomegaly only occurred when mice were treated with known immunosuppressants or MPA. Along with other findings detailed below and from knowledge of inherited defects in purine metabolism (section 1.4.2.1), development of MPA as an immunosuppressive drug began in the early 1980s (Allison and Eugui, 1993a).

1.4.7.1 MPA reduces lymphocyte proliferation

As predicted from its mechanism of action, MPA reduces the proliferation of T and B lymphocytes following stimulation by several mitogens or in mixed lymphocyte reactions (Eugui et al., 1991b). Lymphocyte proliferation in response to tumour or vaccine is attenuated by MPA (Eugui et al., 1991b). Proliferation is limited by MPA induced G_1-S phase cell cycle blockade (Cohn et al., 1999) and although the principal mode of action is cytostatic, MPA can induce apoptosis (Cohn et al., 1999).

1.4.7.2 MPA causes a reduction in antibody formation

MPA reduces B cells number and inhibits antibody formation. This has been demonstrated *in vitro* following activation of human B cells by Staphylococcus protein A sepharose (Allison et al., 1991), tetanus toxoid (Burlingham et al., 1991) or stimulation by interleukin (IL)-4 and IL-13 (Chang et al., 1993). The *in vivo* murine antibody response to sheep erythrocytes was significantly reduced by MPA (Mitsui and Suzuki, 1969, Eugui et al., 1991b).

1.4.7.3 MPA has minimal effect on cytokine production

Several groups have studied the effect of MPA on cytokine production. No inhibition has been demonstrated for IL-2 and IL-2R (Eugui et al., 1991a), IL-4, IL-5, interferon gamma (IFN-γ) and granulocyte macrophage colony stimulating factor (GM-CSF) (Chang et al., 1993) IL-1α, IL-1β, IL-3, IL-6, IL-10, tumor necrosis factor (TNF)-α and TNF-β (Nagy et al., 1993). A 40% reduction in GM-CSF production in response to superantigen was seen following MPA exposure.

1.4.7.4 MPA *in vitro* reduces glycosylation and expression of adhesion molecules

Adhesion molecules, such as selectins and integrins, required for recruitment of leukocytes to sites of inflammation require GTP for their synthesis. By reducing GTP, MPA reduces transfer of mannose to these membrane glycoproteins resulting in reduced expression (Allison and Eugui, 1993b).

1.4.7.5 MPA inhibits nitric oxide production

Nitric oxide (NO) is an important signaling molecule and mediator of the immune response. One of the co-factors required for its synthesis, tetrahydrobiopterin (Moncada et al., 1991) is in turn dependent on GTP for its synthesis. MPA has been shown to reduce production of NO in mouse and rat brain endothelial cells that had been stimulated with a combination of IFN-γ and TNF-α (Senda et al., 1995).

In vivo, MPA has been shown to reduce levels of inducible NO synthase mRNA in the renal cortex of MRL/lpr mice (Yu et al., 2001).

1.4.7.6 MMF in models of allograft rejection

MMF reduces acute rejection in a variety of *in vivo* models. This has been demonstrated for transplanted pancreatic islets (Hao et al., 1990), heart (Tanabe et al., 1994, Fujino et al., 1994, Morris et al., 1990, Morris et al., 1991), kidney (Platz et al., 1991), small bowel (Nakajima et al., 1993) and liver (Bechstein et al., 1993). Many of the studies showed additional benefit from combination therapy with other immunosuppressants. MMF is also effective in the treatment of models of chronic rejection of transplanted aorta (Raisanen-Sokolowski et al., 1994), heart (Morris et al., 1991) and kidney (Azuma et al., 1995).

1.4.8 Clinical studies of MMF

MPA has been used in humans as sodium mycophenolate and a morpholinoethyl ester (MMF) with improved bioavailability (Lee et al., 1990). MPA has also been used in the treatment of several autoimmune diseases including Systemic Lupus Erythematosis (Karim et al., 2002, Moore and Derry, 2006) and Multiple Sclerosis (Michel et al., 2013).

1.4.8.1 Prevention of SOT allograft rejection

An initial trial in 48 primary cadaveric renal transplant recipients receiving MMF (doses 0.1g to 3.5g/day) (Sollinger et al., 1992) showed efficacy in the absence of toxicity with 1-year actuarial graft survival of 95% (Deierhoi et al., 1993). In this study patients also received cyclophosphamide and prednisolone. With escalating doses of MMF, only one patient experienced an adverse event possibly related to the drug (haemorrhagic gastritis). There was a significant correlation between rejection episodes and dose of MMF ($p=0.022$).

Subsequently three multi-centre prospective, randomized, double-blinded placebo controlled trials of MMF for the prevention of acute rejection post renal transplant were performed (European Mycophenolate Mofetil Cooperative Study Group, 1995, Sollinger, 1995, The Tricontinental Mycophenolate Mofetil Renal Transplantation Study Group, 1996). Pooled analysis of 1493 renal transplant recipients from these trials showed that at one year, MMF significantly reduced the incidence of acute rejection. Overall graft survival rates were higher with MMF but did not reach statistical significance (Halloran et al., 1997). These results formed the basis for approval of MMF for prevention of acute rejection in renal transplant recipients.

MMF also exhibits efficacy in the treatment of refractory acute cellular rejection in renal allografts recipients. As discussed in section 1.2, MMF is commonly given in combination with glucocorticoids and other immune suppressants such as calcineurin inhibitors or biological agents.

1.4.9 Safety and tolerability of MMF

Information on the safety and tolerability of MMF is mainly derived from three large clinical trials in renal transplantation (European Mycophenolate Mofetil Cooperative Study Group, 1995, Sollinger, 1995, The Tricontinental Mycophenolate Mofetil Renal Transplantation Study Group, 1996) and one large clinical trial in heart transplantation (Kobashigawa et al., 1998).

MMF is generally well tolerated but can cause gastrointestinal and hematological side effects. The leading adverse gastrointestinal events are diarrhoea, abdominal pain, nausea and vomiting. Leukopenia and anemia are the most frequently encountered hematological adverse events. These adverse events are dose-dependent and are readily reversible upon dose reduction or

discontinuation. As with other immunosuppressive drugs, MMF treatment is associated with a higher risk of opportunistic infection. In particular, there is a tendency for a slight increase in the incidence of tissue-invasive cytomegalovirus infection, especially at the higher dose of 3g/day.

There is no clear evidence for increased incidence of malignancy. Investigation of the effects of different immunosuppressants in a murine model of hepatic metastasis in colon cancer demonstrated reduction in tumour growth in mice receiving therapeutic concentrations of MMF but enhanced tumour growth when ciclosporin or tacrolimus were given (Yokoyama et al., 1995). In patients receiving immune suppression, there is data suggesting reduced rates of malignancy when MMF is used in comparison to other agents. Analysis of registry data in renal transplant recipients has shown a trend towards reduced incidence and a significant increase in time to onset of post-transplant malignancy compared to other immunosuppressive regimen (Robson et al., 2005). Post-transplant Kaposi sarcoma has been shown to regress when immunosuppression was changed from ciclosporin to MMF (Hussein et al., 2000), however another reports suggest an increased risk of Kaposi sarcoma while taking MMF (Eberhard et al., 1999).

1.5 Hypotheses

In this work I will use a retroviral vector to transduce cells with a mycophenolic acid resistant mutant of inosine-5'-monphosphate ($IMPDH2^{R}$) to test the following hypotheses:

1. Transduction with $IMPDH2^{R}$ will confer a selective advantage compared to untransduced cells or cells transduced with wild-type or hypofunctional IMPDH2 in the presence of mycophenolic acid.
2. $IMPDH2^{R}$ will confer a selective advantage due to protection from MPA induced cell cycle blockade and apoptosis.
3. $IMPDH2^{R}$ transduced cells will persist and function *in vivo* during treatment with Mycophenolate Mofetil.

1.6 Aims

1. To generate $IMPDH2^{CS}$, $IMPDH2^{WT}$ and $IMPDH2^{R}$ transduced cells and investigate their phenotype *in vitro* in the presence and absence of MPA.
2. To investigate *in vivo* selection of $IMPDH2^{R}$ versus $IMPDH2^{CS}$ transduced CD8 T cells following antigenic stimulation in the presence or absence of MMF.
3. To investigate the function of $IMPDH2^{R}$ *in vivo* during MMF therapy

Chapter 2: Materials and Methods

2.1 Retroviral Vectors

A retroviral vector (SFG) containing human IMPDH2 with the double-mutation T333I and S351Y in frame with eGFP at its N-terminus ($IMPDH2^{R}$) had previously been cloned and was a gift from Dr Martin Pule, UCL. In order to assess whether any advantage seen in cells transduced with $IMPDH2^{R}$ was due to expression of increased levels of IMPDH2 rather than the resistance phenotype, a plasmid containing a wild-type IMPDH2 gene was cloned from the mutant form ($IMPDH2^{WT}$). Additionally, an enzymatically hypofunctional plasmid ($IMPDH2^{CS}$) was generated by insertional mutagenesis to act as a control. A plasmid containing eGFP alone was also used as a control.

pCl-Eco is a retroviral packaging vector, which is used to enhance retroviral titres produced by Ph-Eco cells by encoding retroviral gag, pol and ecotropic envelope proteins. These were used to generate retroviral supernate for the transduction of murine cells. pCl-Ampho was used with Ph-Ampho cells to generate retroviral supernate for the transduction of human cells.

2.1.1 Generation of wild-type IMPDH2

Using the two sets of overlapping primers (sequences below) we generated two DNA fragments (Figure 2.1A) that were subsequently fused to create a fragment in which the T331I and S351Y mutations had been repaired (Figure 2.1B). This was then cloned back into the original mutant vector using Sbf1/BamHI enzyme digestion of the parental plasmid and PCR fusion to generate a plasmid containing wild-type IMPDH2.GFP fusion (Figure 2.1C). The alignment of Primers 2 and 3 to $IMPDH2^{WT}$ is shown (Figure 2.1D).

Primer 1: 5'-CCCAAGGATCGCGTGCGGGATG-3'

Primer 2: 5'-GTTGCTTGGGGCCGCCCACAGGCCAGCACTTCCTGCGTA ATGCAGATGGAGCCACTTCCCATGC-3'

Primer 3: 5'-CCTGTGGGCGGCCCCAAGCAACAGCAGTGTACAAGGTGT CAGAGTATGCACGGCGCTTTGGTG-3'

Primer 4: 5'-GGCACCAATGTCCTGGCATGAGTGTTG-3'

Figure 2.1 Cloning SFG. eGFP-IMPDH2^{WT}

(A) An SFG plasmid containing an eGFP-IMPDH2^{R} fusion was used as a template for cloning. Two PCR reactions were performed to cover an overlapping 600 base pair region of the IMPDH2 gene contain a BamH1 and Sbf1 restriction site and the 2 mutations S351Y and T333I.

(B) The two overlapping PCR products were separated on a 1% agarose gel and extracted. A secondary PCR reaction using the outermost primers (1 and 4) was performed and the resulting product separated on a 1% agarose gel and extracted.

(C) Both the PCR product and the SFG.eGFP-IMPDH2^{R} plasmid were digested using BamH1 and Sbf1 and a PCR reaction run using primers 2 and 3 to fuse the secondary PCR product into the SFG plasmid. Successful generation of an SFG.eGFP-IMPDH2^{WT} plasmid was confirmed by DNA sequencing.

(D) The sequences of the overlapping primers 2 and 3 and the aligned IMPDH2^{R} sequence are shown.

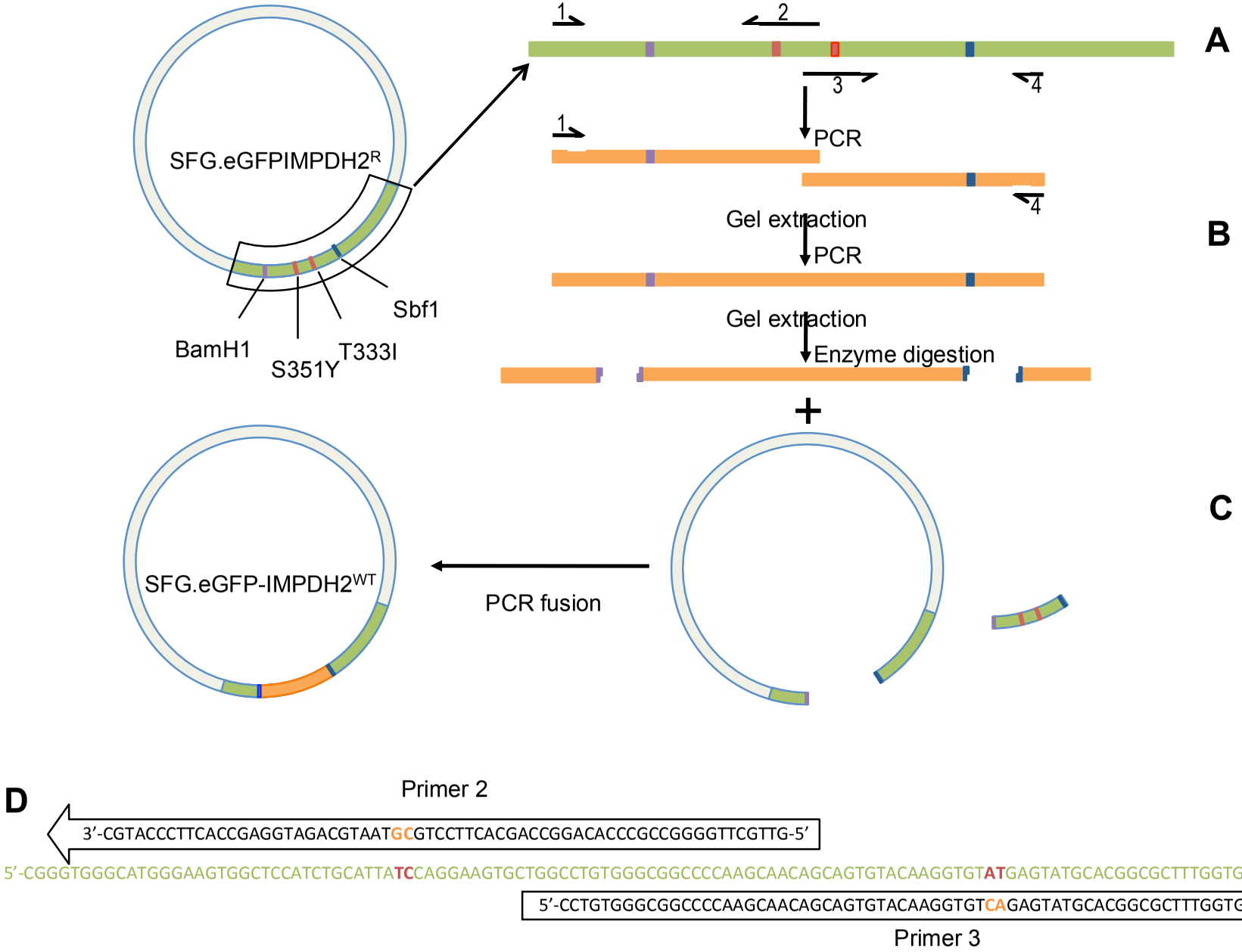

SFG.eGFPIMPDH2R
BamH1
S351Y
T333I
Sbf1
1
2
3
4
A
PCR
Gel extraction
B
PCR
Gel extraction
Enzyme digestion
C
PCR fusion
SFG.eGFP-IMPDH2WT
D
Primer 2
3'-CGTACCCTTCACCGAGGTAGACGTAATGCGTCCTTCACGACCGGACACCCGCCGGGGTTCGTTG-5'
5'-CGGGTGGGCATGGGAAGTGGCTCCATCTGCATTATCCAGGAAGTGCTGGCCTGTGGGCGGCCCCAAGCAACAGCAGTGTACAAGGTGTATGAGTATGCACGGCGCTTTGGTGTTCCG-3'
5'-CCTGTGGGCGGCCCCAAGCAACAGCAGTGTACAAGGTGTCAGAGTATGCACGGCGCTTTGGTG-3'
Primer 3

Figure 2.1

2.1.2 Generation of catalytic-site mutant IMPDH2

Futer *et al* identified a C331A mutation in IMPDH (IMPDH2^{CS}) that results in an enzyme with a K_{cat} <0.00007/s compared to 1.51/s for the wild type enzyme(Futer et al., 2002). I generated this hypofunctional mutant of IMPDH2 by insertional mutagenesis using QuickChange® Mutagenesis XL kit (Agilent) as per the manufacturers instructions (Figure 2.2). The C331A mutant required a two base pair mutation T991G and G992C. Primers (Invitrogen Life Sciences) were designed using the online Stratagene Quickchange primer design program.

5'-GGGAAGTGGCTCCATCGCCATTACGCAGGAAGTG-3'

5'-CACTTCCTGCGTAATGGCGATGGAGCCACTTCCC-3'

A PCR reaction was set up using the two primers highlighted above, pfu turbo-polymerase, the SFG.eGFP-IMPDH2^{WT} plasmid, quick solution, dNTP mix and B˚. The PCR product was incubated in DpnI to digest the parental plasmid but not the unmethylated PCR product. XL-10 Gold cells were transformed using the DpnI treated DNA and then grown on agar plates containing ampicillin. The resultant colony was expanded and DNA extracted using a miniprep kit (Qiagen). Sequencing was performed which confirmed the insertion of the C331A mutation.

Figure 2.2 Cloning SFG.eGFP-IMPDH2^{CS}

Using a QuickChange® Mutagenesis XL kit, a PCR reaction with the placental plasmid SFG.eGFP-IMPDH2^{WT} and mutagenic primers was performed. Parental plasmid was digested with Dpn1, which does not digest the unmethylated PCR product containing the mutagenic primers. XL10 Gold cells were transformed with this mutated plasmid. Successful mutation was confirmed by DNA sequencing.

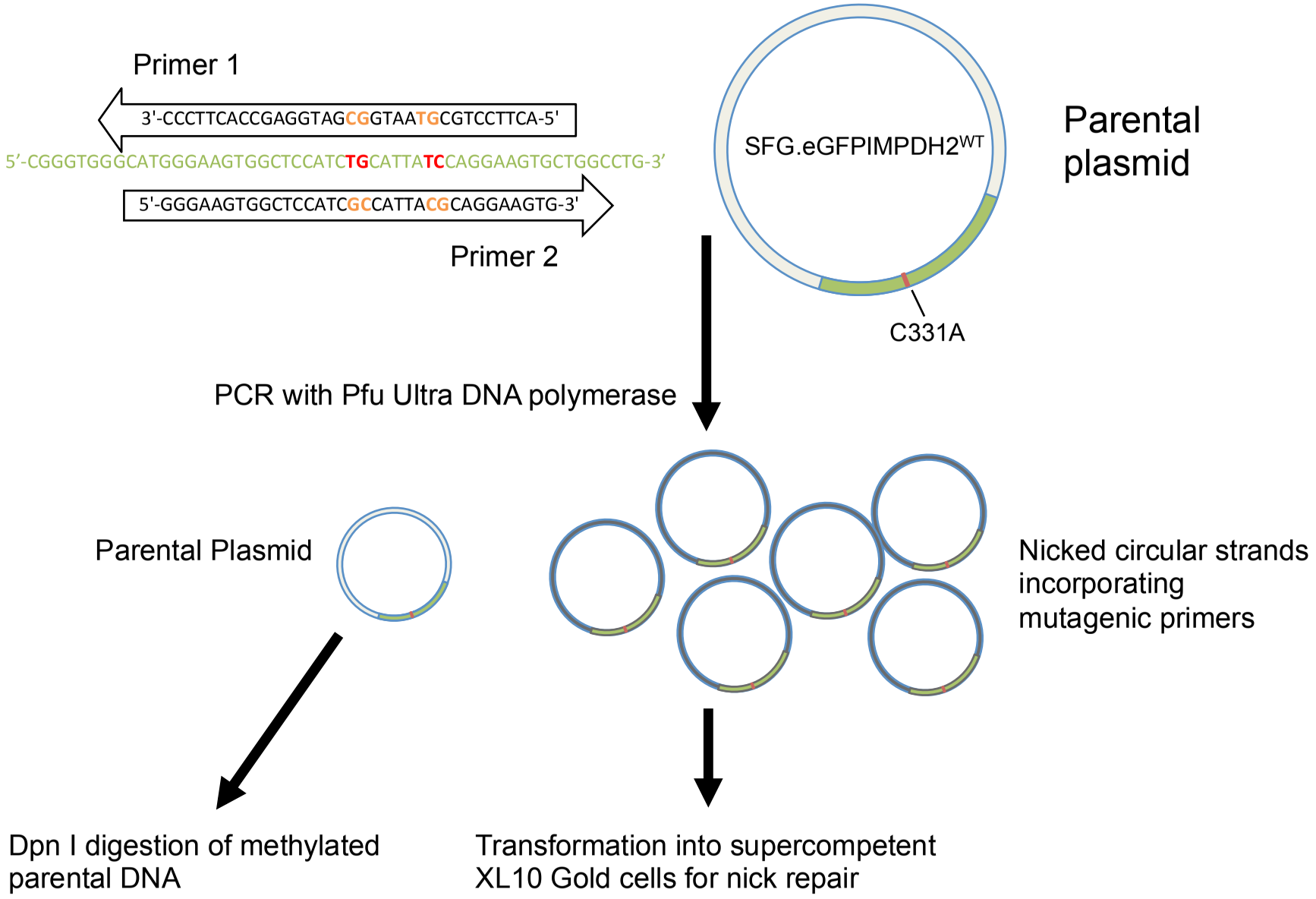

Primer 1
3'-CCCTTCACCGAGGTAGCGGTAATGCGTCCTTCA-5'
5'-CGGGTGGGCATGGGAAGTGGCTCCATCTGCATTATCCAGGAAGTGCTGGCCTG-3'
5'-GGGAAGTGGCTCCATCGCCATTACGCAGGAAGTG-3'
Primer 2
SFG.eGFPIMPDH2WT
C331A
Parental plasmid
PCR with Pfu Ultra DNA polymerase
Parental Plasmid
Nicked circular strands incorporating mutagenic primers
Dpn I digestion of methylated parental DNA
Transformation into supercompetent XL10 Gold cells for nick repair

Figure 2.2

2.2 Cell culture

2.2.1 Murine cells

C57Bl/6, OT1 TCR Tg Rag-/-(Hogquist et al., 1994) (CD45.1, CD45.1xCD45.2 or CD45.2), Thy1.1 (C57Bl/6 background), CD45.1 and CD45.1xCD45.2 mice (C57Bl/6 background) were obtained from breeding colonies established at our on-site animal facility.

2.2.2 Cell lines

The Phoenix Ecotropic (Ph-Eco) Packaging cell line (Nolan Laboratory, Stanford, CA) is an adherent cell line used as the packaging line to generate high concentrations of retroviral particles following transient transfection. Phoenix Eco cells stably express from gag, pol and env the proteins required for packaging, processing, reverse transcription and integration of retroviral genomes, and include selectable markers for gag-pol expression (hygromycin resistance) and env expression (diphtheria toxin resistance). In addition Phoenix Amphotrophic (Ph-Ampho) Packaging cells were used which are very similar to Ph-Eco cells, but their env gene produces supernate that is trophic for human cells.

RMAS cells were used as antigen presenting cells to murine lymphocytes as they do not present endogenous antigen, but after temperature induction express high levels of empty MHC class I on their surface, which can be loaded with peptide (Ljunggren et al., 1990, Esquivel et al., 1992, De Bruijn et al., 1991). A T2 cell line, generated from fusion of a human B-Lymphoblastoid cell line (LCL) and T-LCL, is deficient in transporter associated with protein (TAP) but still express low amounts of MHC class I on the surface of the cells (Salter and Cresswell, 1986, Salter et al., 1985). After loading with peptide these cells

were used in restimulation of TCR transduced human peripheral blood mononuclear cells.

A CD8+ variant of the BW5147 thymoma cell line (BW) was used to assess transduction, confirm phenotype and to ensure larger numbers of analyzable cells (Wegener et al., 1992, Letourneur and Malissen, 1989).

The EG7 cell line was used in *in vivo* tumour experiments. EG7 was generated by transfection of the C57BL/6 (H-2b) derived cell line EL4 with a plasmid containing a complete cDNA copy of the chicken ovalbumin (OVA) mRNA and a neomycin resistance gene which allows for selection of transfected cells by G418 (Moore et al., 1988). EG7 cells form solid tumours in mice (Zhou et al., 1992) and present the immunodominant peptide epitope of $OVA_{257-264}$ (SIINFEKL) in the context of K^b, which is recognised by the OT1 TCR(Shastri and Gonzalez, 1993, Rotzschke et al., 1991).

Adherent cells were cultured in T75 and non-adherent cell lines in T25 or T75 tissue culture flasks (Helena Biosciences, UK). All tissue culture procedures were performed in a class II tissue culture cabinet.

2.2.3 Cell counting and viability

Cells were counted using a disposable Neuberger improved haemocytometer under a light microscope. Cell viability was assessed using 0.4% trypan blue (Sigma); live cells are capable of excluding the trypan blue dye.

2.2.4 Culture media

2.2.4.1 Murine T cells – T cell media

Medium used comprised RPMI-1640 (Roswell Park Memorial Institute - Lonza) with 10% heat inactivated foetal bovine serum (Sigma), 100Units/mL penicillin, 100µg/mL streptomycin (Invitrogen), 2mM L-glutamine (Sigma-Aldrich) and 0.05mM 2-mercaptoethanol.

2.2.4.2 RMAS cells and BW cells – Cell line media

Medium used comprised RPMI-1640 (Roswell Park Memorial Institute - Lonza) with 10% heat inactivated foetal bovine serum (Sigma), 100Units/mL penicillin, 100µg/mL streptomycin (Invitrogen) and 2mM L-glutamine (Sigma-Aldrich).

2.2.4.3 EG7 cells – EG7 media

Medium comprised of DMEM with 10% heat inactivated foetal bovine serum (Sigma) and 2mM L-glutamine (Sigma-Aldrich). G418 (400µg/ml) was added to the culture to select OVA transfected cells.

2.2.4.4 Packaging cell media

Medium used comprised IMDM (Iscove's Modified Dulbecco's Medium - Lonza) with 10% heat inactivated foetal bovine serum (Biowest), 100Units/mL penicillin, 100µg/mL streptomycin (Invitrogen) and 2mM L- glutamine (Sigma-Aldrich).

2.2.5 Cytokines

Interleukin-2 (IL2) (Chiron) was reconstituted in PBS and aliquots stored at -20˚C. IL-7 (R&D) was reconstituted in PBS + 2% Fetal calf serum at 1ng/µl and aliquots stored at -20˚C. IL-15 (Peprotech) was reconstituted in PBS + 2% Fetal calf serum at 10ng/µl and aliquots stored at -20˚C. Concanavalin A (ConA) (Sigma-Aldrich) was reconstituted at 2µg/µl. Aliquots were stored at -20˚C and diluted 1:1000 in culture media for usage.

2.2.6 Mycophenolic acid

Mycophenolic acid (Sigma-Aldrich) was reconstituted in Methanol at 50mg/ml (156mM) and stored at -20˚C.

2.2.7 Peptides

All peptides were obtained from Proimmune, UK. Ovalbumin peptide (SIINFEKL) is presented in the context of H2-K^b. MDM2 peptide was used as a control. Peptides were reconstituted in phosphate buffered saline (PBS) to a stock concentration of 2mM and stored at -20°C.

2.3 Retroviral transduction

2.3.1 Transfection and retroviral particle production

Cells were seeded on tissue culture-treated 10cm dishes at 1.5 x 10^6 in 8mL of culture medium. After 24 hours the medium was replaced with 5ml culture medium and the Phoenix Eco cells transfected using the lipid-based Fugene-HD transfection reagent (Roche) in serum-free Opti-MEM medium with 2.6μg relevant plasmid DNA and 1.5μg pCl Eco DNA. After a further 24 hours the medium was replaced with 5.5ml T-cell medium. 24 hours later this retroviral particle-containing medium was collected and centrifuged at 1500rpm for 5 minutes to remove cellular debris. The retroviral supernatant was then either used directly for transduction of activated murine splenocytes or stored at -80˚C for later use.

2.3.2 Magnetic bead selection and activation of CD8+ T cells

CD8+ T cells were purified from the spleens of C57Bl/6 mice, using anti-CD8α (Ly-2, Miltenyi) MACS beads and LS magnetic columns (Miltenyi). Purification was carried out by positive selection according to manufacturer's instructions. Briefly, up to 2 x 10^8 splenocytes were washed in MACS buffer (2% Bovine Serum Albumin (BSA), 2mM EDTA in PBS) and resuspended in 90μL MACS buffer and 10μL anti-CD8α MACS beads per 10^7 cells. The suspension was incubated for 20 minutes at 4°C. Cells were washed with MACS buffer, resuspended in minimum of 500μL for up to 1x10^8 cells and applied to a prepared LS column in a MACS generated magnetic field. The column was washed with 3 x 3ml of MACS buffer before being removed from the MACS magnet. The CD8+ T cells were then eluted from the column with 5ml of MACS

buffer. Purity was checked by FACS analysis and was consistently greater than 90% for CD8+ T cells (data not shown).

The eluted cells were counted and resuspended at 1.5 x 10^6/ml in T cell media containing 1ng/ml IL-7 and 2µg/mL Concanavalin A (ConA). The cells were incubated for 24-26 hours prior to retroviral transduction

2.3.3 Retronectin

Retronectin (Takara Bio, Japan) was made up as per manufacturers instructions and stored at -20˚C. Retronectin was re-used up to 10 times.

2.3.4 Transduction of CD8+ murine splenocytes

Non-tissue culture treated 24 well plates (Cellstar) were coated with 750µl retronectin/well and incubated at room temperature for 2-3 hours. Retronectin was removed and plates were blocked for 30 minutes at room temperature with filter sterilized 2% BSA/PBS (2.5ml per well). 1 x 10^6 activated splenocytes were re-suspended in 750µl viral supernatant and added to one well of a retronectin-coated plate. For mock transduction, 1 x 10^6 activated splenocytes were re-suspended in 750µl conditioned T-cell media. Cells were centrifuged at 2000g for 90 minutes at 4 ˚C with no brake and then incubated at 37°C, 5% CO_2. On the day following transduction (Day 1), the media in each well was replaced with 2ml fresh T-cell media with IL-2 100u/ml, IL-7 1ng/ml, and IL-15 10ng/ml. On day 3 the cells were aspirated and counted, then resuspended in fresh media at 1 x 10^6/ml. Transduction efficiency was assessed by FACS analysis for GFP.

2.3.5 Maintenance of murine cells in culture

For experiments with long-term *in vitro* culture of primary murine CD8+ cells, media was refreshed on alternate days with IL-2, IL-7, and IL-15 unless otherwise specified. When mycophenolic acid was also present, a working stock solution was made and added at appropriate concentration to media prior to resuspension of cells.

2.3.6 Retroviral Transduction of BW cells

BW cells do not require activation prior to transduction. Following the protocol above, using retronectin, results in very high rates of stable transduction >80%. To test batches of frozen retroviral supernate and for enrichment experiments, 5 x 10^5 BW cells/well were plated into a 96 well plate in 200µl retroviral supernate. Cells were centrifuged at 2000g for 90 minutes at 4 ˚C with no brake and the contents of each well were aspirated, added to 5ml cell line media in a T25 flask and transferred to an incubator. Transduction was assessed 3 days later.

2.3.7 Preparation of Human Peripheral Blood Mononuclear Cells (PBMC)

Human PBMC were generated from cones of donor blood by the protocol detailed below and stored in our institutions Biobank. Blood was layered on top of Lymphoprep (Ficoll) in a 50ml falcon tube at a ratio of 1:2 to the blood sample volume. The falcon was then centrifuged at 1600 rpm for 20 min with no break. The interphase was careful aspirated with a Pasteur pipette into another falcon tube and washed with PBS. The collected cells were typed for HLA-A2 by flow cytometry (only HLA-A2 positive cells used) and then resuspend cells in freezing medium.

2.3.8 Retroviral transduction of human PBMC

Transduction of human PBMC closely follows the protocol used for murine CD8+ splenocytes. Defrosted cells are resuspended at 1 x 10^6/ml in T cell media and activated with OKT3 (CD3 Antibody) at 30ng/ml and IL-2 (Chiron) 600 units/ml 48hours prior to transduction. Retronectin coated plates are used with 750µl viral supernatant per well for 1 x 10^6 activated PBMC. For dual transduction, 750µl of each supernate are used. Unlike with murine cells, the plate is not centrifuged. Approximately 3-4 days following transduction the media is replaced with 2ml fresh T-cell media with IL-2 100u/ml.

2.4 Peptide restimulation

2.4.1 Re-stimulation of transduced murine splenocytes

Following transduction, murine CD8+ T cells were cultured in T-cell media with IL-2, IL-7 and IL-15 for 7 days. On day 6 RMAS were resuspended at 1 x 10^6/ml in a 25-cm^2 non-filter flask. The cap was loosened and flask transferred to an incubator for 5 hours. The cap was fastened and flask transferred to 26˚C water-bath overnight. RMAS cells were resuspended at 1 x 10^6/ml in fresh T cell media with 10μM peptide and incubated for 2 hours. Concurrently, a single cell suspension of C57Bl/6 splenocytes was made to produce feeder cells. The RMAS and feeder cells were irradiated for 14 and 7 minutes respectively. In a 24 well plate, 5 x 10^5 CD8+ T cells, 2 x 10^6 irradiated feeder cells and 1 x 10^5 RMAS cells/ well were suspended in 2ml T cell media + 20/ml Roche IL-2 +/- mycophenolic acid. Wells were assessed daily and split 1:2 with fresh media after 3 days. Analysis was performed 7 days after restimulation.

2.4.2 Re-stimulation of transduced human PBMC

7 days following transduction transduced human PBMC underwent stimulation with peptide loaded feeder cells in a 24 well plate. T2 cells were peptide loaded with EBV $LMP2_{426-434}$ CLGGLLTMV peptide or CMV $pp65_{495-503}$ NLVPMVATV peptide for 2 hours. 2 x 10^5 T2 cells per well were incubated with 100μM peptide and then irradiated for 14 minutes. Additionally, PBMC from the same donor were defrosted and irradiated for 7 minutes to act as feeder cells, with 2 x 10^6 required for each well. The irradiated peptide-loaded T2 cells and PBMC feeders were cultured with 5 x 10^5 transduced cells in a total of 2ml T cell media with 2 units/ml of IL2 (Roche). The colour of media was monitored and media replenished as required. Restimulation was repeated every 7-10 days.

For measurement of cytokine production, 5 x 10^5 transduced PBMC were cultured with 5 x 10^5 peptide loaded T2 cells in 200µl media overnight in a 96 well round bottomed plate. Supernate was aspirated and stored at -20˚C prior to measurement.

2.5 Measurement of secreted IL-2 and IFN-γ by enzyme-linked immunosorbent assay (ELISA)

The concentration of IL-2 and IFN-γ in culture supernatants was determined using OptEIA ELISA kits (BD – IL-2 555190, IFN-γ 555142) according to the manufacturer's instructions summarised below. The reagents used were supplied in the kit unless otherwise specified and included:

- Coating buffer – 0.1M sodium carbonate
- Assay diluent – PBS with 10% Fetal Bovine Serum (FBS)
- Wash buffer – PBS with 0.05% Tween-20
- Working detector – biotin-linked detection antibody and streptavidin-linked horseradish peroxidase
- BD substrate solution (BD – 51-2607/6)
- Stop solution (1M sulphuric acid – Fisher Scientific, Loughborough, UK).

96 well Nunc-Immuno™ polystyrene Maxisorp ELISA flat bottom plates were coated with 100µL of appropriate capture antibody diluted 1:250 in coating buffer and incubated overnight at 4°C. The plates were aspirated and washed three times. Wells were block with 100µl Assay Diluent and incubated for 1 hour at room temperature. The plates were aspirated and washed three times. 100µl standard or sample (diluted in assay diluent) was added to each well and the plate incubated for 2 hours at room temperature. The plates were aspirated

and washed five times. 100µl prepared Working Detector was added to each well and incubated for 1 hour at room temperature. The plates were aspirated and washed seven times. 100µl TMB One-Step Substrate Reagent was added to each well and the plate incubated in the dark for 30 minutes at room temperature (no plate sealer). 50µl Stop Solution was added to each well and the plate read at 450 nm within 30 minutes, λ correction 570nm using a Multiskan EX photometer (Thermo Scientific, Basingstoke, UK)

2.5.1 Standards preparation

Lyophilised recombinant IL-2 (BD – 554603) and lyophilised recombinant IFN-γ (BD – 554617) were dissolved in assay diluent and diluted to 1000pg/ml. Successive doubling dilutions standards were prepared in duplicate from 1000pg/mL to 3.125pg/mL.

For each assay a standard curve was derived by log-log regression analysis using Excel 2011 (Microsoft).

2.6 Flow cytometry

Samples were acquired on an LSRII or Fortessa flow cytometer (BD Biosciences, USA) and analysed using FlowJo software (Tree Star, Oregon). All buffers were kept at 4°C.

2.6.1 Cell surface staining

0.25-0.5x10^6 cells/well 96 well plate were washed with FACS buffer (2% FBS in PBS) and resuspended in 50µL of FACS buffer with appropriate amount of fluorochrome-conjugated antibody, determined by prior titration. Suspensions were incubated in the dark for 15-20 minutes at 4°C, washed in 150µL then 200µL of FACS buffer and resuspended in 200µL of FACS buffer. Multimer staining was performed in 50µl FACS buffer at room temperature, prior to washes and further surface staining.

2.6.2 Fluorescent labelled antibodies

Table 2.1 contains a list of all the fluorescent-labelled antibodies used in the experiments detailed within this work. In addition, in most experiments, cells were positive for GFP and fluorescence from this was detected in FL-1.

Table 2.1 Fluorescent-labelled antibodies

Antigen	Fluorochrome	Clone	Supplier	Catalog No
B220	PerCP-Cy5.5	RA3-6B2	eBioscience	45-0452-82
B220	BIOTIN	RA3-6B2	eBioscience	13-0452-85
CD19	FITC	ID3	BD	553785
CD335	PE-Cy7	29A1.4	eBioscience	25-3351-80
CD4	PerCP	RM4-5	BD	553052
CD44	PE	IM7	Pharmigen	553134
CD45.1	APC-eFluor 780	A20	eBioscience	47-0453-82
CD45.2	PerCP-Cy5.5	104	BD	552950
CD62L	APC	MEL-14	BD	553152
CD8a (Ly-2)	APC	53-6.7	BD	553035
CD8a (Ly-2)	PE	53-6.7	eBioscience	12-0081-83
CD8a (Ly-2)	V450	53-6.7	BD	560469
FC BLOCK		93	eBioscience	14-0161-85
IFN-GAMMA	APC	4S.B3	BD	554413
NK1.1	PE	PK136	eBioscience	12-5941-81
NK1.1	PE-Cy7	PK136	eBioscience	47-5941-80
NK1.1	BIOTIN	PK136	BD	553163
STREPTAVIDIN	APC		BD	554067
TCR–β constant	APC	H57-597	eBioscience	17-5961-81
THY-1.1 (CD90.1)	PE-Cy7	HIS51	eBioscience	25-0900-82
THY-1.2 (CD90.2)	PE-Cy7	53-2.1	eBioscience	25-0902-81
VA2	PE	B20.1	BD	553289
Vα2	BIOTIN	B20.1	BD	553287
Vβ5.1/5.2	FITC	MR9-4	BD	553189
Vβ5.1/5.2	PE	MR9-4	BD	553190
Human CD4	APC-Cy7	RPA-T4	BD	557871
Human CD8	PE-Cy7	RPA-T8	BD	557746
$LMP2_{426-434}$ (CLGGLLTMV) HLA A*02:01 pentamer	PE		ProImmune	F042-2A

2.6.3 Detection of Apoptosis – Annexin V staining

Apoptosis was detected with an Annexin V APC staining kit (BD). Briefly, 1.5-3 x 10^5 cells were surface stained as above and then washed twice in cold PBS. Resuspended in 50µL of Annexin V Binding buffer with 2µL Propidium iodide (PI) and 1.25µL Annexin V APC. Suspensions were incubated at room temperature, protected from light for 20 minutes. Each sample was made up to 200 µL with Annexin V Binding buffer and analysed by flow cytometry within 1 hour.

2.6.4 Analysis of Cell Cycling

Cells were replated at 5 x 10^5/well 96 well plate in media containing 10µM Bromodeoxyuridine (BrdU; BD). Following 30-60 minutes incubation at 37˚C, 5% CO_2 cells were washed with FACS buffer and surface stained as above. Staining for BrdU and 7-Aminoactinomycin D (7-AAD) was carried out using a BrdU-APC kit (BD Pharmingen). Cells were fixed and permeabilised, treated with DNAse and then stained for BrdU incorporation and with 7-AAD as per the kit instructions.

2.7 *In vivo* Experiments

Females of 8-10 weeks of age were used as splenocyte donors for *in vitro* and *in vivo* experiments; females of 10 weeks or older were used as recipients. All procedures were carried out in accordance with United Kingdom Home Office regulations. To assess cell turnover, BrdU was added to the drinking water to a final concentration of 0.8M and replenished every 3-4 days. Irradiated mice received 21 days of Baytril starting 7 days prior to irradiation. Following irradiation mice were fed millet and wet feed in order to maintain weight.

2.7.1 Mycophenolate mofetil

Mycophenolate mofetil (Cellcept) was reconstituted in either 5% dextrose (Baxter) or a resuspension vehicle (CMC) containing 0.9% sodium chloride, 0.5% Tween 80, 0.5% benzyl alcohol and 0.8% carboxymethylcellulose. In 5% dextrose MMF was administered within 60 minutes of reconstitution, aliquots of MMF in CMC where stored at 4˚C for up to 5 days prior to administration. MMF was administered daily via intraperitoneal (i.p.) injection in 200μl diluent for 7 to 21 days. Dose was adjusted according to body weight.

2.7.2 Peptide

Peptide was administered *in vivo* via subcutaneous (s.c.) injection at the base of the tail. Prior to injection an emulsion of peptide and incomplete Freund's adjuvant (IFA; Sigma) was made at a concentration of 200μM and 100μl was injected.

2.7.3 Procedures

In cell transfer experiments, cells were injected intravenously (i.v.) via the tail vein suspended in 200μl phosphate buffered saline.

EG-7 cells were suspended in 100μl of matrigel (BD) and remained on ice until immediately prior to s.c. injection. Measurement of tumour was with callipers to record perpendicular measurements of diameter.

Blood was taken by capillary action following of the tail tip. Following collection the tail tip was cauterised with silver nitrate.

Mice were checked 5 minutes after all procedures.

2.8 Statistical analysis

GraphPad Prism version 6.0 for Mac OsX (GraphPad Software, San Diego, USA) was used for statistical analyses. P values of 0.05 or less were considered statistically significant. Comparisons between groups were carried out using two-tailed, paired Student's t-test for *in vitro* experiments and Mann-Whitney test for *in vivo* experiments.

Chapter 3: *In vitro* functional analysis of IMPDH2^{R} transduced cells

3.1 Introduction

By culturing a murine neuroblastoma cell line in increasing concentrations of mycophenolic acid (MPA), Hodges *et al.* developed a cell line resistant to MPA (Hodges et al., 1989). This resistance was shown to be due to cells exhibiting both gene amplification of inosine-5'-monophosphate dehydrogenase (IMPDH), resulting in a 200-500 fold increase in protein levels as well as mutations in isoform 2 of the IMPDH gene (IMPDH2). Of the four mutations identified, one was present in the parental cell line and another did not result in an amino acid change (Lightfoot and Snyder, 1994). The remaining 2 mutations, T333I and S351Y ($IMPDH2^R$), are found in the nicotinamide adenine dinucleotide (NAD) binding pocket of IMPDH2, which is also the site of MPA binding. Purification of the mutant enzyme showed an unchanged Michaelis constant for inosine monophosphate (IMP) but a 4-fold increase in the Michaelis constant for NAD. The dissociation constant (K_i) of $IMPDH2^R$ for MPA was shown to be 2400 fold higher than wild-type IMPDH2 ($IMPDH2^{WT}$).

Intracellular nucleotide levels govern the ability of a cell to proliferate due to DNA replication. Adequate GTP levels are also important to enable normal cellular signalling through G-coupled proteins. When GTP is depleted, a cell will initially undergo cell cycle arrest and with increased depletion undergo apoptosis. As previously described, MPA inhibits the *de* novo synthesis of guanosine nucleotides. MPA has been shown to block cell cycle progression from G_1 to S phase and with increasing concentrations of drug causes cell death by apoptosis (Cohn et al., 1999).

MPA has been shown to act via inhibition of IMPDH (Franklin and Cook, 1969), which is required for *de novo* synthesis of GTP. A second pathway (salvage pathway), where HGPRTase converts guanine into guanosine monophosphate that can subsequently be metabolised to GTP and dGTP, is present in almost all cell types. The salvage pathway helps to protect most cell types from the effects of MPA. Lymphocytes express little HGRPTase, relying almost entirely on IMPDH for synthesis of guanosine nucleotides and therefore MPA is lymphocyte specific. In addition, when activated and requiring increased levels of GTP there is an isoform shift from primarily IMPDH1 to IMPDH2 (Nagai et al., 1992). IMPDH2 is 4 times more sensitive to MPA than IMPDH1 (Carr et al., 1993) and MPA has most effect against proliferating cells.

3.2 Aims

- To generate plasmids encoding the wild-type and enzymatically hypofunctional (Futer et al., 2002) IMPDH2 genes
- To demonstrate the selective advantage conferred to cells by IMPDH2^{R} transduction in the presence of MPA
- To investigate the protective effect of IMPDH2^{R} against MPA induced apoptosis and cell cycle arrest
- To test the ability of IMPDH2^{R} cells cultured in MPA to produce cytokines in response to antigenic stimulation

3.3 Results

In vitro investigation of IMPDH2^{R} phenotype: Murine cell line

To investigate the selective advantage conferred by IMPDH2^{R}, I used the vectors generated in figures 2.1 (pp. 75) and 2.2 (pp. 78) to produce retroviral supernate for transduction. I first transduced a CD8+ variant of the murine BW5147 thymoma cell line with IMPDH2^{CS}, IMPDH2^{WT} or IMPDH2^{R}. Additionally, an SFG vector containing eGFP alone was also used. Initial transduction efficiency ranged from 21-58%. Transduced BW cells were FACS sorted to obtain pure populations of GFP expressing cells (purity >95%), which stably expressed the transduced construct during prolonged culture. These were used in the experiments outlined in sections 3.3.1, 3.3.3 and 3.3.4.

3.3.1 IMPDH2^{R} expression increases the IC_{50} for MPA compared to IMPDH2^{CS}, while IMPDH2^{WT} confers an intermediate phenotype.

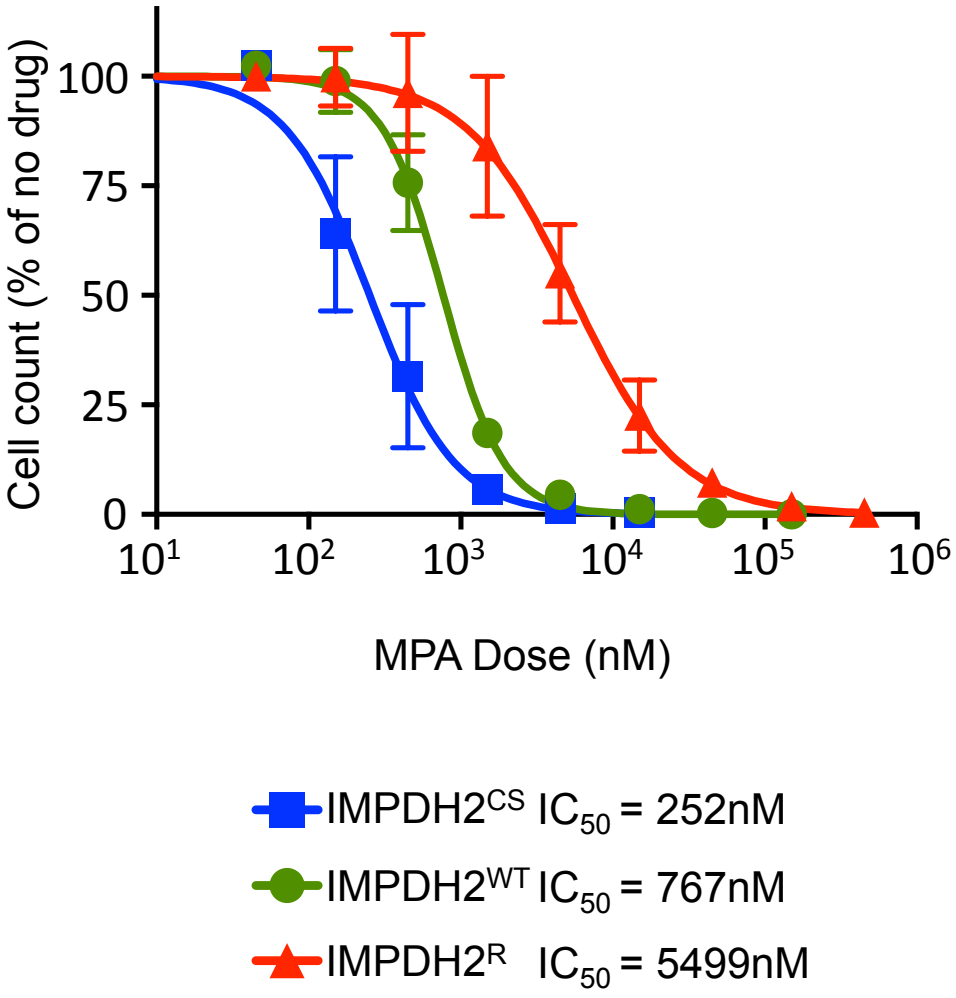
Sorted transduced BW cells were cultured in concentrations of MPA from 0-1mM. Cell counts were performed at 48 hours and normalised to the count without drug. Dose response curves were modelled and are shown with IC_{50} calculated for each IMPDH2 construct (Figure 3.1). Cells transduced with IMPDH2^{CS} showed suppression of cell growth at similar doses of MPA as untransduced BW cells seen in pilot experiments (data not shown) with a calculated IC_{50} of 252nM. IMPDH2^{R} transduced cells continued to expand, compared to baseline, up to MPA concentrations several log higher than IMPDH2^{CS} with a significantly increased IC_{50} calculated at 5499nM (Extra sum-of-squares F test; F value CS v R = 351.4 $p<0.0001$). IMPDH2^{R} transduced

cells also maintained similar cell expansion with or without MPA to a MPA concentration $>1_{log}$ higher than IMPDH2^{CS} transduced cells. IMPDH2^{WT} demonstrated an intermediate phenotype with a calculated IC_{50} 767nM, significantly greater than IMPDH2^{CS} and less than IMPDH2^{R} (F values CS v WT = 103.7, WT v R = 332.4; both $p<0.0001$).

This demonstrates an advantage, in terms of cell expansion, for IMPDH2^{R} transduced BW cells cultured in MPA.

Figure 3.1 Transduced BW dose response

BW cells were transduced with IMPDH2^{CS}, IMPDH2^{WT} or IMPDH2^{R} and FACS sorted for GFP. 1 x 10^6 cells were suspended in 5ml media with MPA at concentrations 0, 45, 150, 450, 1500, 4500, 15000, 45000, 150000 and 450000nM. Cell counts were taken at 48 hours and normalised to the cell count in the absence of drug. Data from 3 independent experiments was combined and plotted as cell count at each concentration as a percentage of count in the absence of drug, with mean and standard deviation plotted. Dose response curves for the 3 constructs were modelled in GraphPad Prism. The modelled IC_{50} for each construct is shown.



3.3.2 IMPDH2^{R} expression confers a selective advantage during MPA exposure compared to un-transduced cells

As previously shown (Sangiolo et al., 2007, Yam et al., 2006), I hypothesised that IMPDH2^{R} would confer a selective advantage to transduced cells in comparison to untransduced cells when cultured in MPA. Unsorted transduced BW cells were cultured in the presence or absence of two concentrations of MPA (450nM and 4500nM). FACS was performed at baseline and at 48 hours to ascertain percentage of GFP positive cells. Example plots indicative of 4 independent experiments are shown (Figure 3.2A). Cell counts were performed and the fold change in transduced cells calculated and normalised to the fold change without drug (figure 3.2B).

Cells transduced with GFP alone or IMPDH2^{CS} exhibited a significant decrease in transduced cells at both concentrations of MPA (paired t-test GFP 0 v 450 p=0.0334; G0 v 4500 p=0.0014; CS 0 v 450 p=0.0148; CS 0 v 4500 p=0.001; Means GFP 0 MPA=2.216, 450nM MPA=1.171, 4500nM MPA=0.01223, IMPDH2^{CS} 0 MPA=2.285, 450nM MPA=1.157, 4500nM MPA=0.006385). Changes in transduced cell number were not different between the two constructs at either dose. IMPDH2^{WT} transduced cell numbers were non-significantly decreased at both doses (WT 0 v 450 p=0.1517; v 4500 p=0.0894; Means 0 MPA=2.265, 450nM MPA=2.172, 4500nm MPA=0.508).

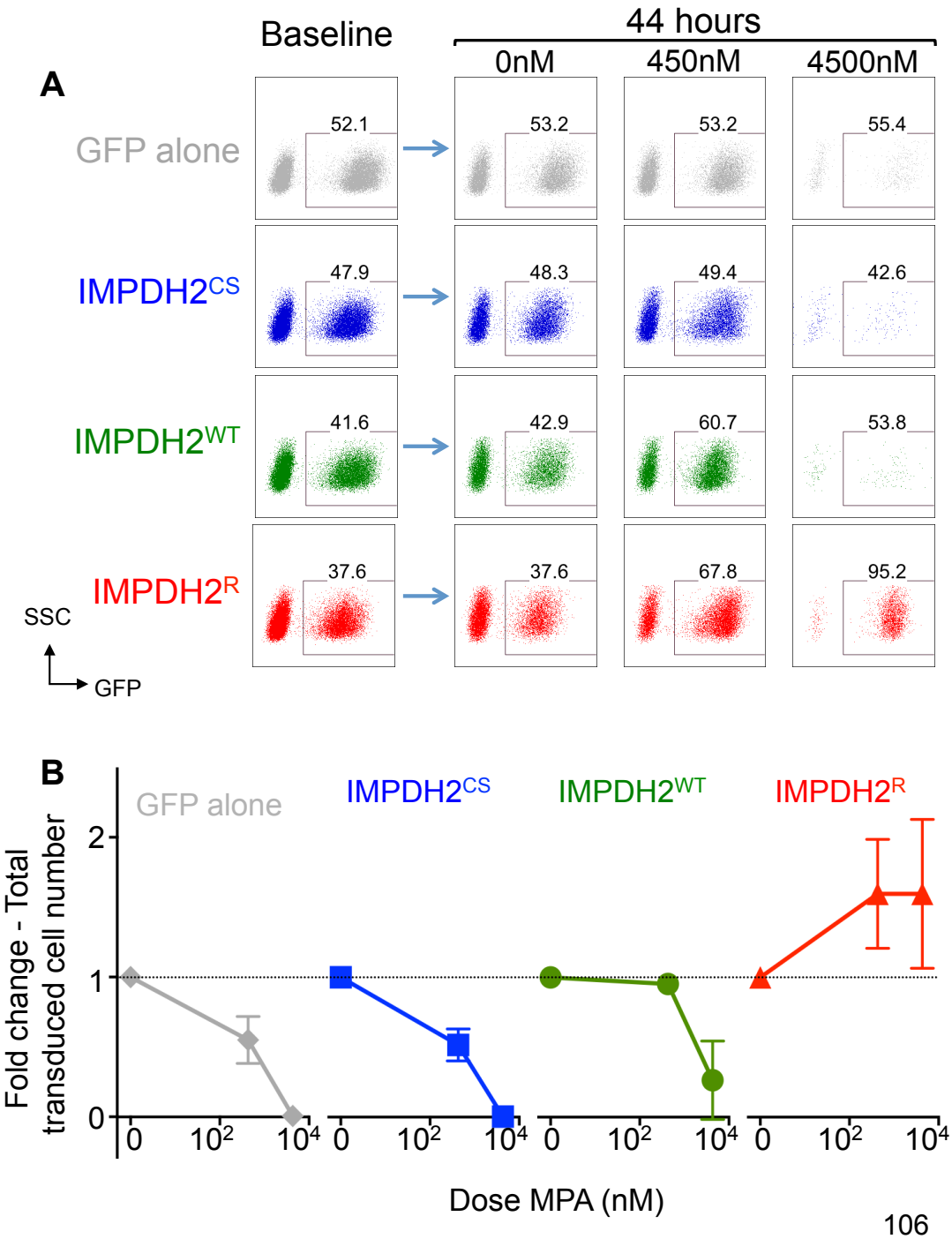
Only IMPDH2^{R} resulted in a fold increase in transduced cells significant at the lower but not higher dose of MPA used (paired t-test R 0 v 450 p=0.0465; R 0v 4500 p=0.0905; Means 0 MPA=2.369, 450nM MPA=3.722, 4500nM MPA=3.684). At both doses of MPA, IMPDH2^{R} transduced cells were selected to significantly greater extent than GFP alone or IMPDH2^{CS} transduced cells

(paired t test R450 v GFP450 p=0.0065; R4500 v GFP4500 p=0.0033; R450 v CS450 p=0.0051; R4500 vs CS4500 p=0.0032). Compared to $IMPDH2^{WT}$ transduced cells there was a significant increase at 450nM but significance was lost at 4500nM (R450 v WT450 p=0.0465; R4500 v WT4500 p=0.0905).

These data demonstrate that $IMPDH2^{R}$ confers transduced cells with a selective advantage over untransduced cells. The number of transduced cells increases to a greater extent with MPA exposure than without. This is due to death of untransduced cells reducing competition for transduced cells.

Figure 3.2 BW cells transduced with IMPDH2^{R} are selected when cultured in MPA

1.5 x 10^{6} transduced BW cells were cultured in 10ml media containing 0, 450 or 4500nM MPA. After 44 hours cell counts were performed and FACS analysis for GFP performed. (A) FACS plots shown were gated on viable cells and are representative of 4 independent experiments. (B) Data are summarised for each group as fold change in the total number of transduced cells normalised to observed change with no drug.



3.3.3 IMPDH2^{R} transduced BW cells overcome MPA-induced cell cycle blockade

I explored potential mechanisms by which IMPDH2^{R} could confer a selective advantage. Because MPA is known to induce cell cycle arrest, I investigated the ability of transduced cells to progress through the cell cycle by staining for incorporated bromodeoxyuridine (BrdU) and deoxynucleic acid (DNA). Sorted transduced cells were cultured in a range of MPA concentrations for 20 hours. An equal number of cells from each condition were then cultured with BrdU (+/- MPA as appropriate) for 20 minutes and then stained for BrdU and 7-Aminoactinomycin D (7-AAD). Example plots (Figure 3.3A) and summary data (Figure 3.3B) are shown.

Cells that are dead or undergoing apoptosis (>G_1) are located in the gate to the extreme left of each plot. Cells in G_0/G_1 have not undergone DNA replication during BrdU exposure and are BrdU negative with an intermediate 7-AAD fluorescence corresponding to a diploid cell. During S phase (top gate), BrdU is incorporated and as DNA is duplicated the 7-AAD signal increases up to twice the baseline level. Cells in G_2 and M phase have high 7-AAD because they have double the number of chromosomes prior to cell division but are BrdU negative because DNA replication has occurred prior to BrdU exposure.

The example plots (figure 3.3A) show that in the absence of MPA, approximately 50% of cells are in S phase and <5% of cells in <G_1 during the 20 minutes of BrdU exposure. When MPA 4500nM is added, IMPDH2^{CS} transduced cells show a reduction in cells in S phase with an associated increase in cells in G_1 phase or that were dead. IMPDH2^{R} transduced cells

maintain similar percentages of cells within each stage of the cell cycle while IMPDH2^{WT} has an intermediate appearance.

Data summarising the effects on cell cycling for each construct at several MPA concentrations is shown separated into plots for $<G_1$, G_0/G_1, S and G_2+M phases (figure 3.3B). A significant increase in the percentage of cells in $<G_1$ phase occurred for both IMPDH2^{CS} transduced cells (paired t-test CS 0 v 1500 p=0.0382) and IMPDH2^{WT} transduced cells (paired t-test WT 0 v 4500 p=0.287). The percentage of cells in $<G_1$ phase was greater for IMPDH2^{CS} than both IMPDH2^{R} and IMPDH2^{WT} except at the highest investigated dose (paired t-test CS450 v WT450 p=0.0492, v R450 p=0.0434; CS1500 v WT1500 p=0.0237, v R1500 p=0.005; CS4500 v WT4500 p=0.102, v R4500 p=0.0354). IMPDH2^{WT} showed significantly increase percentages of cells in $<G_1$ compared to IMPDH2^{R} (paired t-test WT v R 450nM p=0.0172, 1500nM p=0.0317, 4500nM p=0.0365).

G_0/G_1 percentages did not significantly increase for IMPDH2^{CS} and IMPDH2^{R} but there was a significant increase at the highest dose of MPA for IMPDH2^{WT} transduced cells (paired t-test WT0 v 4500 p=0.0165). This indicates that cells are accumulating in G_0/G_1 phase.

At the highest concentration of MPA investigated, there is clear maintenance of the percentage of cells in S phase for IMPDH2^{R} transduced cells compared to those cultured without MPA i.e. preservation of cells entering the cell cycle (Paired t-test R0 v R4500 p=0.706). IMPDH2^{CS} or IMPDH2^{WT} transduced cells show a marked reduction in the percentage of cells in S phase, occurring at a lower dose of MPA for IMPDH2^{CS} (paired t-test CS0 v 1500 p=0.0482; CS0 v 4500 p=0.0351; WT 0v v 1500 p=0.3846, WT 0 v 4500 p=0.0423).

Compared to IMPDH2^{CS}, a higher percentage of IMPDH2^{R} transduced cells are in S phase at both 1500 and 4500nM MPA (paired t-test CS1500 v R1500 p=0.0479; CS4500 v R4500 p=0.0248). More IMPDH2^{WT} transduced cells are in S phase at the lower but not higher concentration of MPA (paired t-test CS1500 v WT1500 p=0.0412, CS4500 v WT4500 p=0.0999). There is a non-significant trend to reduction in IMPDH2^{WT} compared with IMPDH2^{R} (paired t-test WT1500 v R 1500 p=0.3877, WT4500 v R4500 p=0.0709). G_2 + M phase percentages remained similar with all doses of MPA and all three IMPDH2 constructs.

These data show that IMPDH2^{R} protects BW cells from MPA induced cell cycle arrest. IMPDH2^{WT} confers protection from MPA at low concentrations of MPA but the effect is lost as MPA concentration increases.

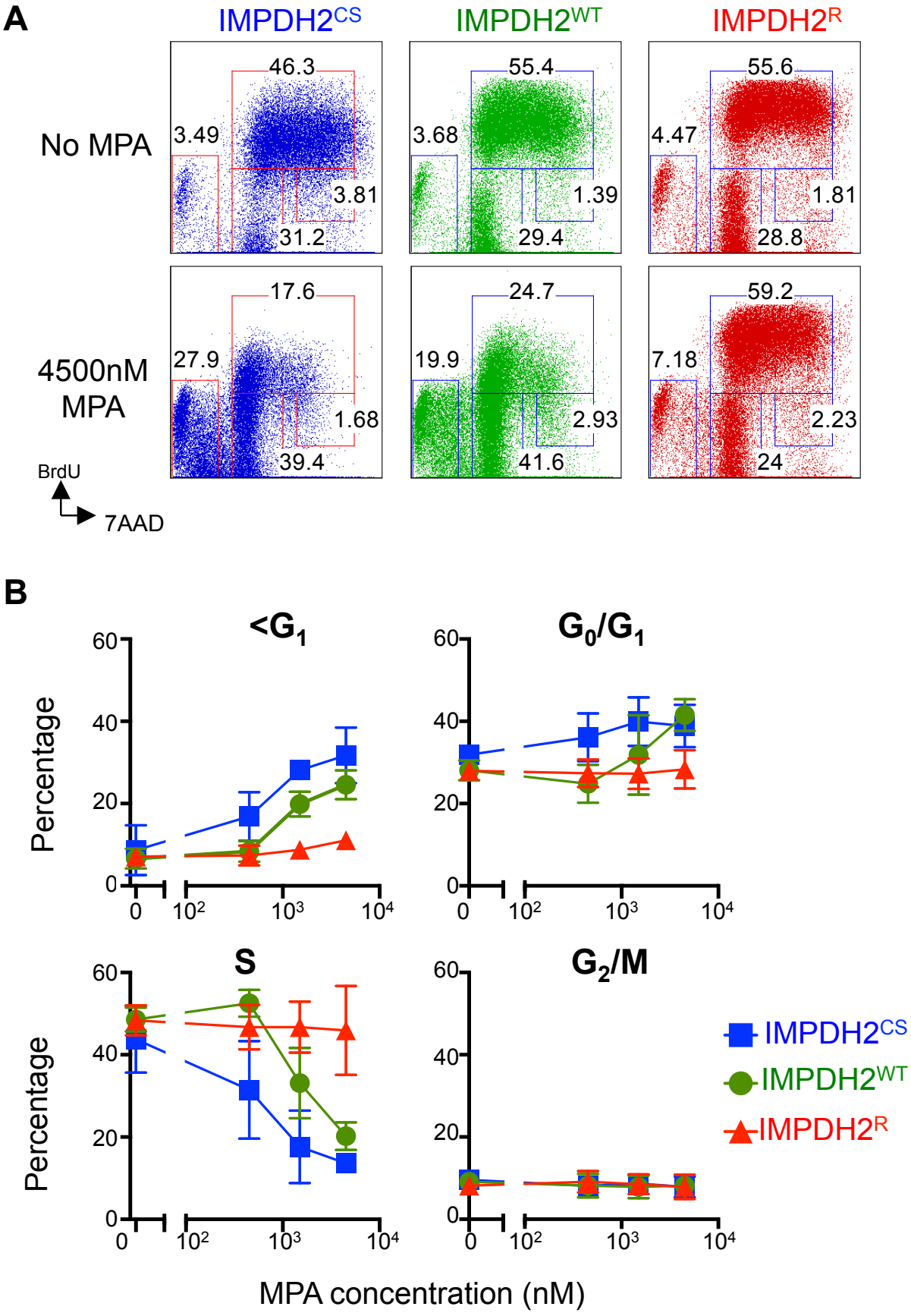
Figure 3.3 IMPDH2^{R} transduced BW cells overcome MPA-induced cell cycle blockade

5 x 10^6 transduced sorted BW cells were cultured in 10ml media with 0, 450, 1500, 4500nM MPA for 20 hours. 1x10^6 cells from each condition were transferred to a 96 well plate and cultured in media containing BrdU and the appropriate concentration of MPA for 20minutes. Cells were then stained for BrdU and 7-AAD using a BrdU staining kit (BD).

(A) Example plots indicative of 3 independent experiments are shown. The plots are otherwise ungated and show the percentages of cells in G_0/G_1 (bottom right), S (top), G_2+M (lower right) and $<G_1$ phases (bottom left).

(B) Data are summarised to show the percentage of cells in each phase of the cell cycle at each dose of MPA.

Figure 3.3


A
IMPDH2CS
IMPDH2WT
IMPDH2R
No MPA
4500nM MPA
46.3
3.49
3.81
31.2
55.4
3.68
1.39
29.4
55.6
4.47
1.81
28.8
17.6
27.9
1.68
39.4
24.7
19.9
2.93
41.6
59.2
7.18
2.23
24
BrdU
7AAD
B
<G1
G0/G1
S
G2/M
Percentage
MPA concentration (nM)
IMPDH2CS
IMPDH2WT
IMPDH2R

3.3.4 IMPDH2^{R} transduced BW cells are protected against MPA-induced apoptosis

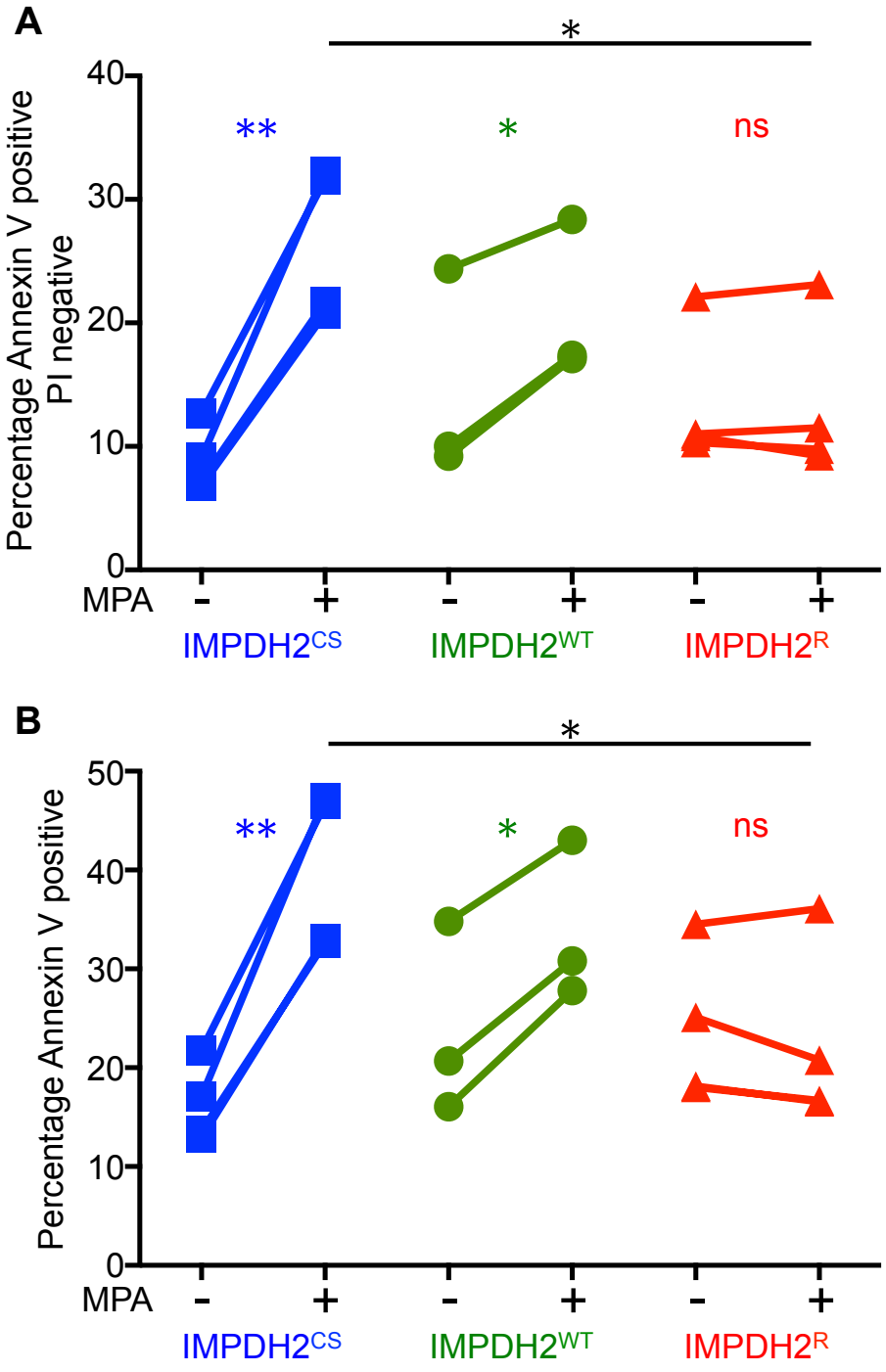
Following cell-cycle arrest, further reduction in IMPDH activity by MPA induces apoptosis. I investigated whether IMPDH2^{R} protects against MPA-induced apoptosis, by culturing transduced BW cells with or without MPA and staining for Annexin V and propidium iodide (PI) at both baseline and after 20 hours. Apoptosis is accompanied by a loss of membrane phospholipid asymmetry, resulting in the exposure of phosphatidylserine (PS) on the cell surface. Annexin V covalently binds to exposed PS and if conjugated to a fluorescent label, can be detected by FACS(Koopman et al., 1994). Of note, cells undergoing apoptosis decrease in size and therefore plots are not pre-gated prior to analysis

The percentage of apoptotic cells (figure 3.4A) and all apoptotic and dead cells (figure 3.4B) are shown. IMPDH2^{CS} transduced cells exhibited a significant increase in apoptosis when exposed to MPA (paired t-test p=0.0037; Mean No MPA=8.9%, MPA=26.6%) that accounted for most of the change in total apoptotic and dead cells (paired t-test p=0.0027; Mean No MPA=16.4%, MPA=39.9%). IMPDH2^{WT} transduced cells cultured with MPA also underwent increased apoptosis (paired t-test p=0.0343; Mean No MPA=14.5%, MPA=21.0%), which again accounted for the majority of the total annexin V positive cells (paired t test p=0.0102; Mean No MPA=23.86%, MPA=33.87%). MPA did not increase the apoptotic or total annexin V positive percentage of IMPDH2^{R} transduced cells (paired t-test apoptosis p=0.815; Mean No MPA=13.6%, No MPA=13.4%; Total annexin V positive p=0.3273; Mean No MPA=23.94%, MPA=22.52%).

There was a significant increase in apoptosis for IMPDH2^{CS} cells compared to IMPDH2^{R} cells (paired t-test p= 0.015). Apoptotic percentage was not different for IMPDH2^{WT} cells compared to IMPDH2^{CS} or IMPDH2^{R} transduced cells exposed to MPA (paired t-test CS v WT p=0.604; WT v R p=0.434).

Figure 3.4 IMPDH2^{R} transduction protects BW cells from MPA-induced apoptosis

5 x 10^{6} transduced sorted BW cells were cultured in 10ml media with or without MPA (4500nM) for 20 hours. 2.5x10^{5} cells were stained with Annexin V and PI as per the Annexin V staining kit (BD). The percentage of apoptotic cells (Annexin V positive PI negative cells) is plotted for each of 3 independent experiments (A). All annexin V positive cells are also plotted (B).



In vitro investigation of IMPDH2^{R} phenotype: Primary murine cells

3.3.5 Antigen dependent selection

To investigate the effects of IMPDH2^{R} in primary cells, I used a model antigen system where all cells express the same T cell receptor (TCR). CD8 T cells from OT1 (Ovalbumin (OVA) TCR 1) TCR transgenic Rag$^{-/-}$ mice were transduced with retrovirus and rested for one week. The OT1 TCR is specific for the chicken $OVA_{257-264}$ peptide SIINFEKL presented in the murine H2-K^{b}. To provide an antigenic stimulus, I used a cell line (RMAS) that expresses class I molecules devoid of peptide at the cell surface, when pre-cultured at low temperature (22˚C), and can be loaded with exogenous peptide(Ljunggren et al., 1990). RMAS were loaded with either relevant (SIINFEKL) or irrelevant (MDM2, a ubiquitous cell surface protein) peptide and irradiated. Irradiated splenocytes, acting as feeder cells, were cultured with transduced OT1 CD8 and the irradiated RMAS in media containing interleukin 2 in the presence or absence of MPA (450 or 1500nM). Media was checked daily and wells were split as necessary, with media refreshed on all wells at the same time. Selection was investigated by FACS analysis (GFP expression) at both baseline and 7 days after stimulation. Three independent experiments were performed and example plots and summary data shown for relevant peptide (Figure 3.5) and irrelevant peptide (Figure 3.6).

Of note, cells cultured with relevant peptide loaded RMAS cells required more media changes than those with irrelevant peptide and when cell counts were performed had a much greater number of trypan blue stained dead cells. When cultured in MPA, very few cells transduced with IMPDH2^{CS} or IMPDH2^{WT}

remain alive at day 7, which is reflected in the example plots shown (Figure 3.5A). This is in contrast to IMPDH2^{R} transduced cells, which persist and exhibit strong selection for transduced cells.

Comparing the fold change in transduced cells (figure 3.5B), in the absence of MPA there is similar expansion of transduced cells with all 3 vectors. IMPDH2^{CS} transduced cells are significantly reduced In the presence of MPA (paired t-test p=0.0191; Mean - No MPA=2.27, MPA=0.35). IMPDH2^{WT} transduced cells exhibit a non-significant reduction in number in MPA compared to no drug and remain static over baseline (paired t-test p=0.074; Mean - No MPA=2.68, MPA=1.00). By contrast, IMPDH2^{R} transduced cells are significantly increased in the presence of MPA over both baseline and cells cultured in the absence of drug (paired t-test p=0.011; Mean - No MPA=2.326, MPA=4.852).

IMPDH2^{CS} cells are significantly reduced in comparison to both IMPDH2^{WT} and IMPDH2^{R} transduced cells when cultured with MPA (paired t-test p= 0.047 and 0.006 respectively). IMPHD2^{WT} numbers are significantly reduced compared to the expansion seen with IMPDH2^{R} (paired t-test p= 0.016).

When cultured with irrelevant peptide loaded RMAS cells (figure 3.6), a similar trend in selection is seen. MPA was added at both the concentration used with relevant peptide and a higher concentration (1500nM). In contrast to stimulation with relevant peptide, IMPDH2^{CS} transduced cells expand in 450nM of MPA and are only significantly reduced at the higher concentration (paired t-test 0 v 450 p=0.1672, 0 v 1500 p=0.0498; Mean No MPA=3.02, MPA 450nM=2.11, 1500nM=0.516). IMPDH2^{WT} transduced cells increase over baseline even at the higher concentration of MPA but a non-significant trend to

reduced expansion with MPA is seen (paired t test 0 v 1500 p=0.0958; Mean No MPA=3.886, 1500nM MPA=1.231). IMPDH2^{R} transduced cells exhibit non-significantly increased expansion at both doses of MPA (paired t-test 0 v 450 p=0.0552, 0 v 1500 p=0.0514; Mean No MPA=3.656, 450nM MPA=6.3, 1500nM MPA=5.723). None of the differences between transduced groups achieved significance at either dose of MPA used (paired t-test MPA 450nM CS v WT p=0.0675, CS v R p=0.0612, WT v R 0.0618; MPA 1500nM CS v WT p=0.0714, CS v R p=0.0553, WT v R 0.0655).

Comparing stimulation with relevant versus irrelevant antigen, there were no significant differences. At the 450nM concentration of MPA, there was a non-significant trend to greater fold change of IMPDH2^{CS} transduced cells with irrelevant antigen compared to relevant (paired t-test p=0.0673; Mean Relevant antigen=2.11, Irrelevant antigen=0.3549).

To investigate whether cells with more copies of the transduced gene are preferentially selected, I analysed the median fluorescence intensity (MFI) for GFP (Figures 3.5C and 3.6B). When stimulated with cognate peptide, IMPDH2^{R} transduced cells exhibit a non-significant increase in GFP MFI following MPA exposure than cells transduced with IMPDH2^{CS} or IMPDH2^{WT} (paired t-test CS v R p=0.0675; WT v R p=0.0633; CS v WT p=0.2626; Mean CS=320, WT=858, R=21567). A non-significant trend was also seen at the higher concentration of MPA when irrelevant peptide was used (paired t-test CS v R p=0.1718; WT v R p=0.1751; CS v WT p=0.0768; Mean CS=329, WT=437, R= 3835). There was no significant difference between relevant and irrelevant antigen for any of the IMPDH2 groups.

Figure 3.5 IMPDH2^{R} transduced OT1 CD8 are selected when stimulated by cognate peptide in the presence of MPA

CD8 T cells were MACS sorted from OT1 TCR Tg Rag$^{-/-}$ spleens and stimulated overnight with Con-A and IL7 and subsequently transduced with IMPDH2^{CS}, IMPDH2^{WT} or IMPDH2^{R}. A group was mock transduced to determine GFP gating on subsequent FACS analysis. The cells were rested for 6 days prior to restimulation with peptide-loaded RMAS cells. Each group was cultured with relevant peptide with or without MPA for 7 days, Cells were counted and FACS analysis for CD8 and GFP performed.

(A) Example plots indicative of 3 independent experiments are shown. The plots are pre-gated on viable lymphocytes.

(B) Results are summarised from 3 independent experiments and given as a fold change in the number of transduced cells.

(C) The median fluorescence intensity of GFP is plotted for each group in the presence or absence of MPA. Mean and standard deviation are given.

Figure 3.5

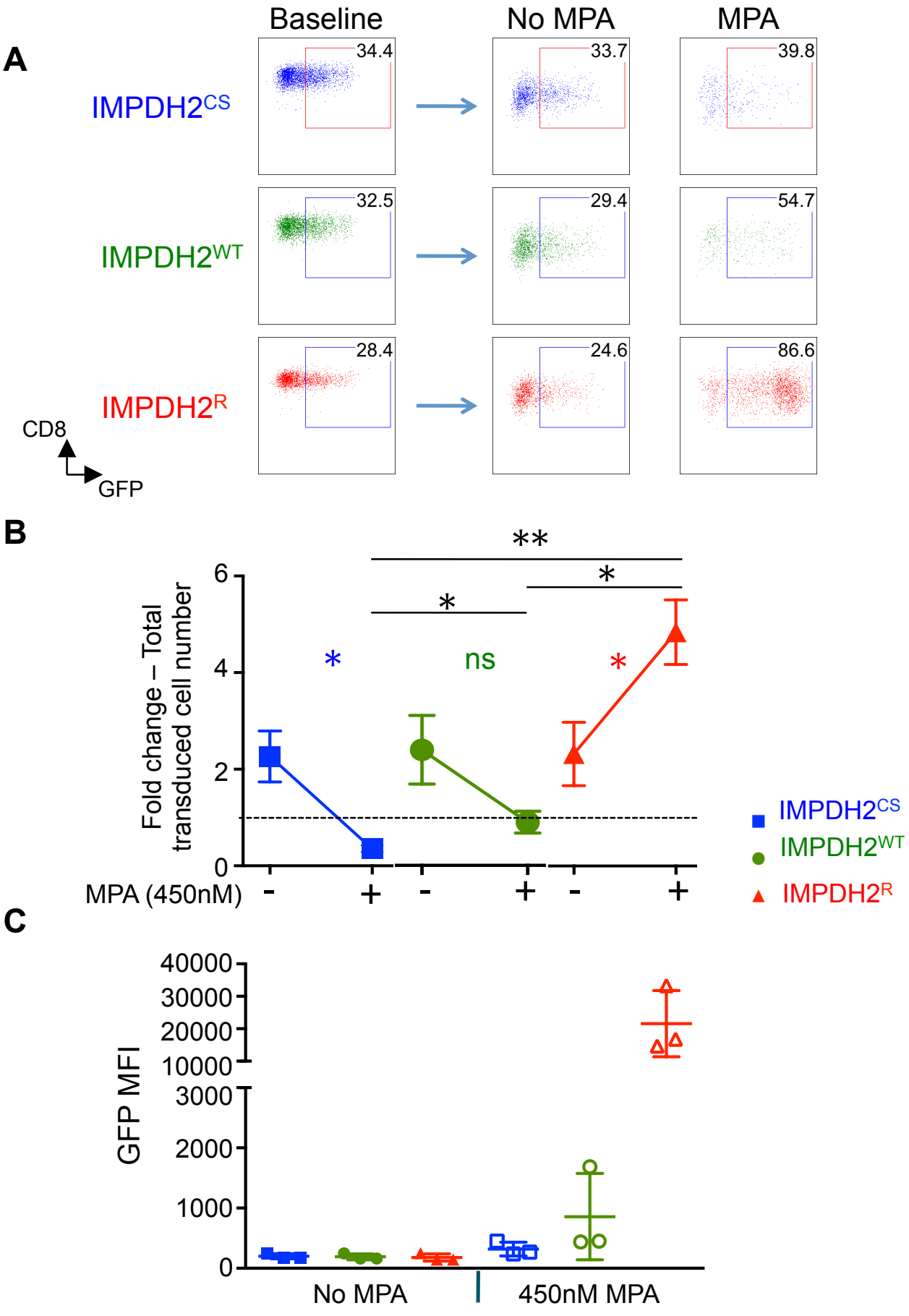

A
Baseline
No MPA
MPA
IMPDH2CS
IMPDH2WT
IMPDH2R
34.4
33.7
39.8
32.5
29.4
54.7
28.4
24.6
86.6
CD8
GFP
B
Fold change – Total transduced cell number
MPA (450nM)
ns
IMPDH2CS
IMPDH2WT
IMPDH2R
C
GFP MFI
No MPA
450nM MPA

Figure 3.6 IMPDH2^{R} transduced OT1 CD8 are selected when exposed to non-cognate peptide in the presence of MPA

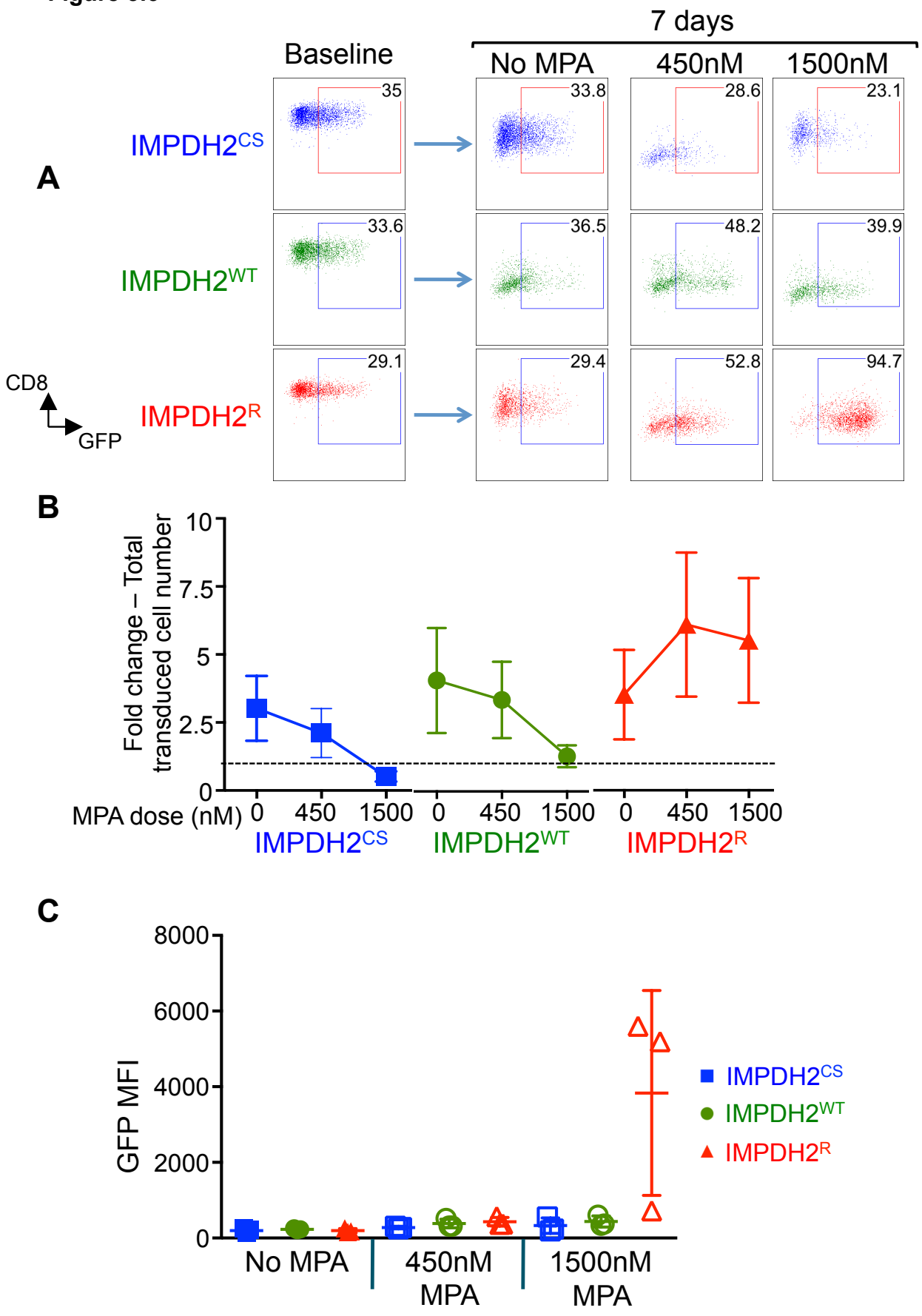
CD8 T cells were MACS sorted from OT1 TCR Tg Rag$^{-/-}$ spleens and stimulated overnight with Con-A and IL7 and subsequently transduced with IMPDH2^{CS}, IMPDH2^{WT} or IMPDH2^{R}. A group was mock transduced to determine GFP gating on subsequent FACS analysis. The cells were rested for 6 days prior to restimulation with peptide-loaded RMAS cells. Each group was cultured with irrelevant peptide with or without MPA for 7 days, Cells were counted and FACS analysis for CD8 and GFP performed.

(A) Example plots indicative of 3 independent experiments are shown. The plots are pre-gated on viable lymphocytes.

(B) Results are summarised from 3 independent experiments and given as a fold change in the number of transduced cells.

(C) The median fluorescence intensity of GFP is plotted for each group in the presence or absence of MPA. Mean and standard deviation are given.

Figure 3.6


A
Baseline
7 days
No MPA
450nM
1500nM
IMPDH2CS
IMPDH2WT
IMPDH2R
35
33.8
28.6
23.1
33.6
36.5
48.2
39.9
29.1
29.4
52.8
94.7
CD8
GFP
B
Fold change – Total transduced cell number
10
7.5
5
2.5
0
MPA dose (nM) 0 450 1500 0 450 1500 0 450 1500
IMPDH2CS
IMPDH2WT
IMPDH2R
C
GFP MFI
8000
6000
4000
2000
0
No MPA
450nM MPA
1500nM MPA
IMPDH2CS
IMPDH2WT
IMPDH2R

3.3.6 IMPDH2^{R} transduced cells are progressively selected under antigen independent conditions

I hypothesised that selection would occur following antigenic stimulation of transduced cells exposed to MPA. Therapeutic IMPDH2^{R} transduced cells will not always be stimulated by antigen. I therefore investigated whether IMPDH2^{R} transduced cells are selected under conditions more analogous to homeostatic proliferation. I hypothesised that selection would occur when transduced murine cells were maintained in culture by common gamma chain cytokines. C57Bl6/J CD8 T cells were transduced and after 3 days cultured with IL-2, IL-7 and IL-15 in the presence or absence of MPA. Cell counts and percentage of GFP positive cells were assessed over the next 10 days. The fold change in transduced cell number is summarised in figure 3.7.

In the absence of MPA, the cell number changes are not significantly different at any time point for IMPDH2^{CS}, IMPDH2^{WT} or IMPDH2^{R} transduced cells. There is an initial expansion (two-fold) in the number of transduced cells by day 3 followed by contraction to approximately 75% of baseline at day 10 (figure 3.7A). The reduction in transduced cell is a result of the GFP positive percentage reducing in the absence of MPA while the total number of cells under these conditions remains or static or reduces slightly.

At the lower concentration of MPA used (450nM), the same concentration used in experiments outlined for cognate antigen in section 3.3.5, IMPDH2^{R} but not IMPDH2^{CS} or IMPDH2^{WT} transduced cells undergo significant expansion (paired t-test compared to Baseline IMPDH2^{CS} p=0.1739, IMPDH2^{WT} p=0.1329, IMPDH2^{R} p=0.0285; Mean IMPDH2^{CS}=1.255, IMPDH2^{WT}=1.536, IMPDH2^{R}=2.140). Transduced cell numbers reduce in all groups, with

IMPDH2^{R} transduced cells remaining at higher levels than IMPDH2^{CS} transduced cells with IMPDH2^{WT} transduced cells in between. At day 10, IMPDH2^{CS} transduced cells are significantly reduced in MPA with greater reduction with higher MPA concentration (paired t-test 0 v 450nM MPA p=0.0129, 0 v 1500nM MPA p=0.0103, 450 v 1500nM MPA 0.0236; Mean No MPA 0.75, 450nM MPA 0.44, 1500nM MPA 0.12). IMPDH2^{WT} transduced cells at the same time point are not significantly reduced at the lower dose of MPA compared to no drug but are reduced by the higher dose (paired t-test 0 v 450nM MPA p=0.9843, 0 v 1500nM MPA p=0.0277, 450 v 1500nM MPA 0.0286; Mean No MPA 0.69, 450nM MPA 0.69, 1500nM MPA 0.45). IMPDH2^{CS} transduced cells are not significantly reduced compared to IMPDH2^{WT} transduced cells (paired t-test p=0.0765) but IMPDH2^{R} transduced cells are higher than both the other constructs (paired t-test IMPDH2^{R} v IMPDH2^{CS} p=0.0367, v IMPDH2^{WT} p=0.045).

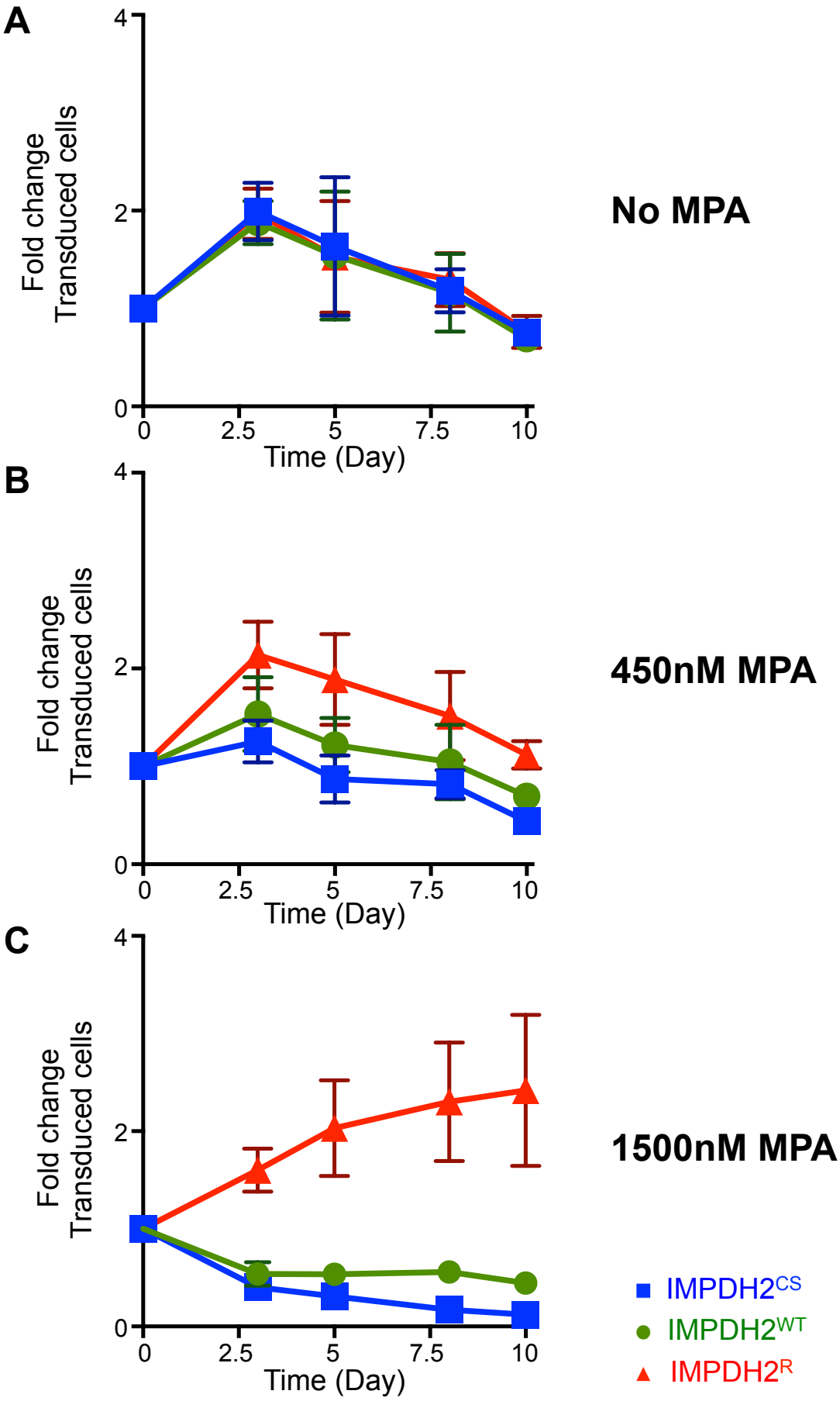
At the higher concentration of MPA (1500nM), both IMPDH2^{WT} and IMPDH2^{CS} transduced cells are significantly reduced by day 3 (paired t-test compared to baseline, IMPDH2^{CS} p=0.0074, IMPDH2^{WT} p=0.0206). In contrast, IMPDH2^{R} numbers increase steadily over the course of MPA exposure and are significantly increased over baseline by day 3 (paired t-test p=0.0226). At day 10, IMPDH2^{CS} transduced cells are significantly reduced compared to IMPDH2^{WT} and IMPDH2^{R} transduced cells (paired t-test IMPDH2^{CS} v IMPDH2^{R} p=0.0362, IMPDH2^{CS} v IMPDH2^{WT} p=0.0109) but IMPDH2^{R} was not significantly increased compared to IMPDH2^{WT} (paired t-test p=0.0544).

Figure 3.7 IMPDH2^{R} transduced cells are progressively selected in antigen independent conditions in the presence of MPA.

CD8 T cells were MACS sorted from C57Bl6/J splenocytes and stimulated with ConA and IL7. They were transduced with IMPDH2^{CS}, IMPDH2^{WT} or IMPDH2^{R} and subsequently rested for 3 days. Cells were cultured in media containing IL-2, IL-7 and IL-15 in the presence or absence of MPA. Media was refreshed every other day. Transduction was assessed by FACS for GFP and monitored over 10 days of culture. Data from 3 independent experiments summarising the fold change in transduced cell number are shown for cells culture in the absence of MPA (A), 450nM MPA (B) and 1500nM MPA (C). Mean and standard deviation are shown.

Red = IMPDH2^{R}; Green = IMPDH2^{WT}; Blue = IMPDH2^{CS}

Figure 3.7


A
B
C
Fold change Transduced cells
Time (Day)
0
2
4
0
2.5
5
7.5
10
No MPA
450nM MPA
1500nM MPA
IMPDH2CS
IMPDH2WT
IMPDH2R

In vitro investigation of IMPDH2^{R} phenotype: Human Peripheral Blood Mononuclear Cells

3.3.7 Selection and expansion of TCR redirected PBMC

Having demonstrated both antigen dependent and independent selection of IMPDH2^{R} transduced murine CD8 T cells, I investigated whether IMPDH2^{R} would provide a selective advantage to cells whose specificity had been redirected by transduction with a specific TCR. I used human peripheral blood mononuclear cells (PBMC) prepared from donor buffy coats and co-transduced LMP2 TCR (specific for the EBV peptide CLG) along with either IMPDH2^{CS}, IMPDH2^{WT} or IMPDH2^{R}. After transduction cells were rested in culture for 7 days and then underwent 3 stimulations with peptide loaded T2 cells at 7-10 day intervals in the presence or absence of MPA. Selection of TCR and GFP was determined by FACS analysis. Example plots and summary data (Figure 3.8) are shown.

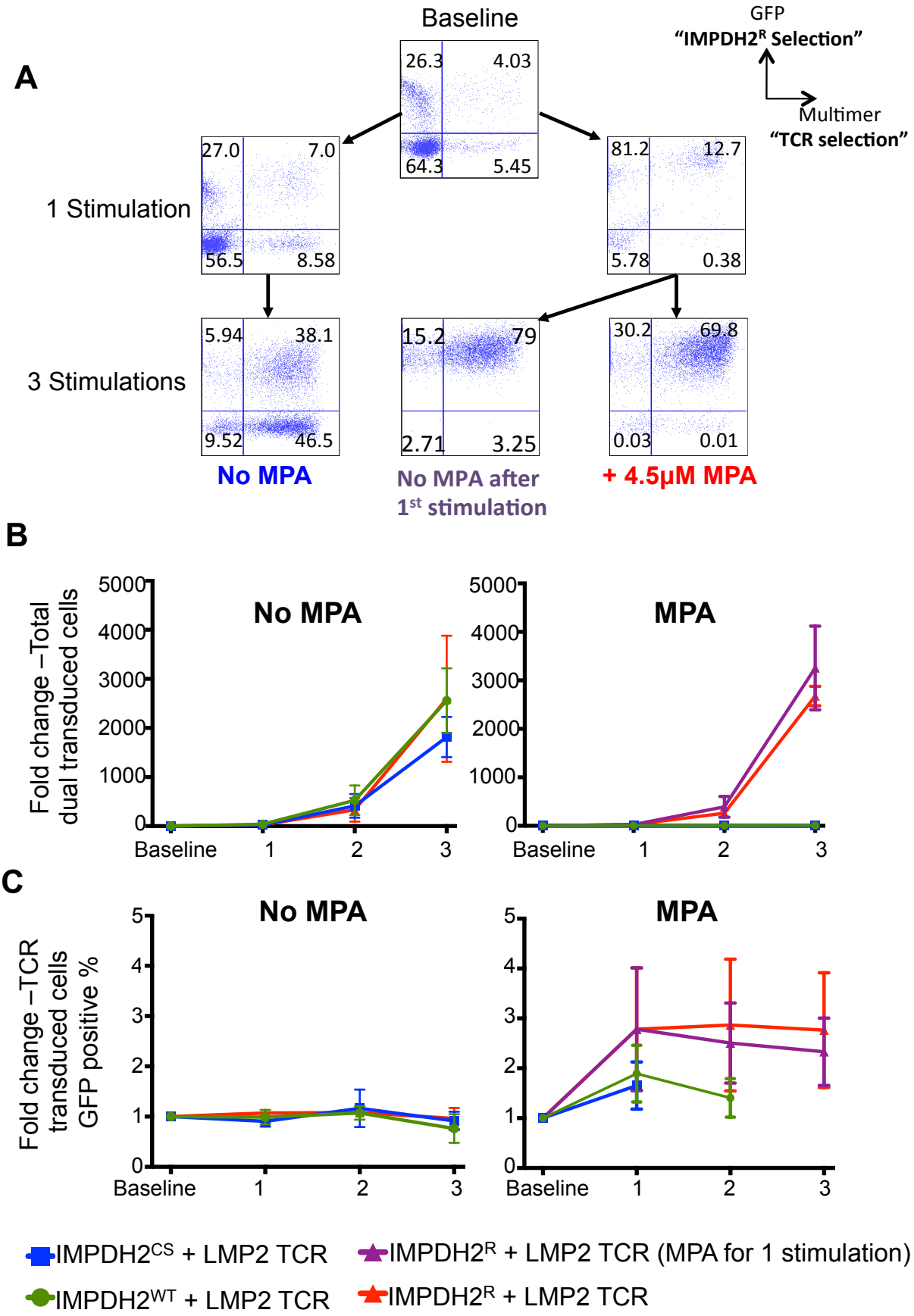
Dual transduced cells were only a minor population at baseline, accounting for approximately 5% of CD8 T cells. Repeated stimulation with peptide resulted in selection and expansion of TCR transduced cells in all groups. When cultured with MPA, cells transduced with IMPDH2^{CS} and IMPDH2^{WT} underwent high levels of cell death and insufficient cells were available in both groups to complete more than the first 1 or 2 stimulations. IMPDH2^{R} was strongly selected when exposed to MPA with the majority of remaining cells GFP positive prior to the second stimulation. Overall the total number of dual LMP2 TCR and IMPDH2^{R} transduced cells was similar with or without MPA, however the purity was higher when cultured under the selective pressure of MPA. In second and subsequent stimulations, I cultured a group of cells without MPA

that had been MPA selected during the first stimulation. The IMPDH2^{R} selection reduced slightly but was not significantly different (Figure 3.8B).

Figure 3.8 Human PBMC dual transduced with IMPDH2^{R} and LMP2 TCR are selected during restimulation with cognate peptide and MPA.

Human donor peripheral blood mononuclear cells were prepared from buffy coats and stimulated with OKT3 and IL-7 prior to transduction with LMP2 TCR and either IMPDH2^{CS}, IMPDH2^{WT} or IMPDH2^{R}. One week after transduction, FACS for GFP and pentamer for LMP2 TCR determined the baseline percentage of transduced cells. Cells were then stimulated with peptide-loaded T2, human PBMC and IL2 in the presence or absence of MPA. **(A)** Example plots gated for viable CD8 lymphocytes are shown at baseline and after 1 and 3 rounds of stimulation for cells transduced with both LMP2 TCR and IMPDH2^{R}. Summary data from 3 independent experiments are given with **(B)** the fold change in total dual transduced cell number and **(C)** the fold change in the percentage of TCR positive cells expressing GFP.

Figure 3.8

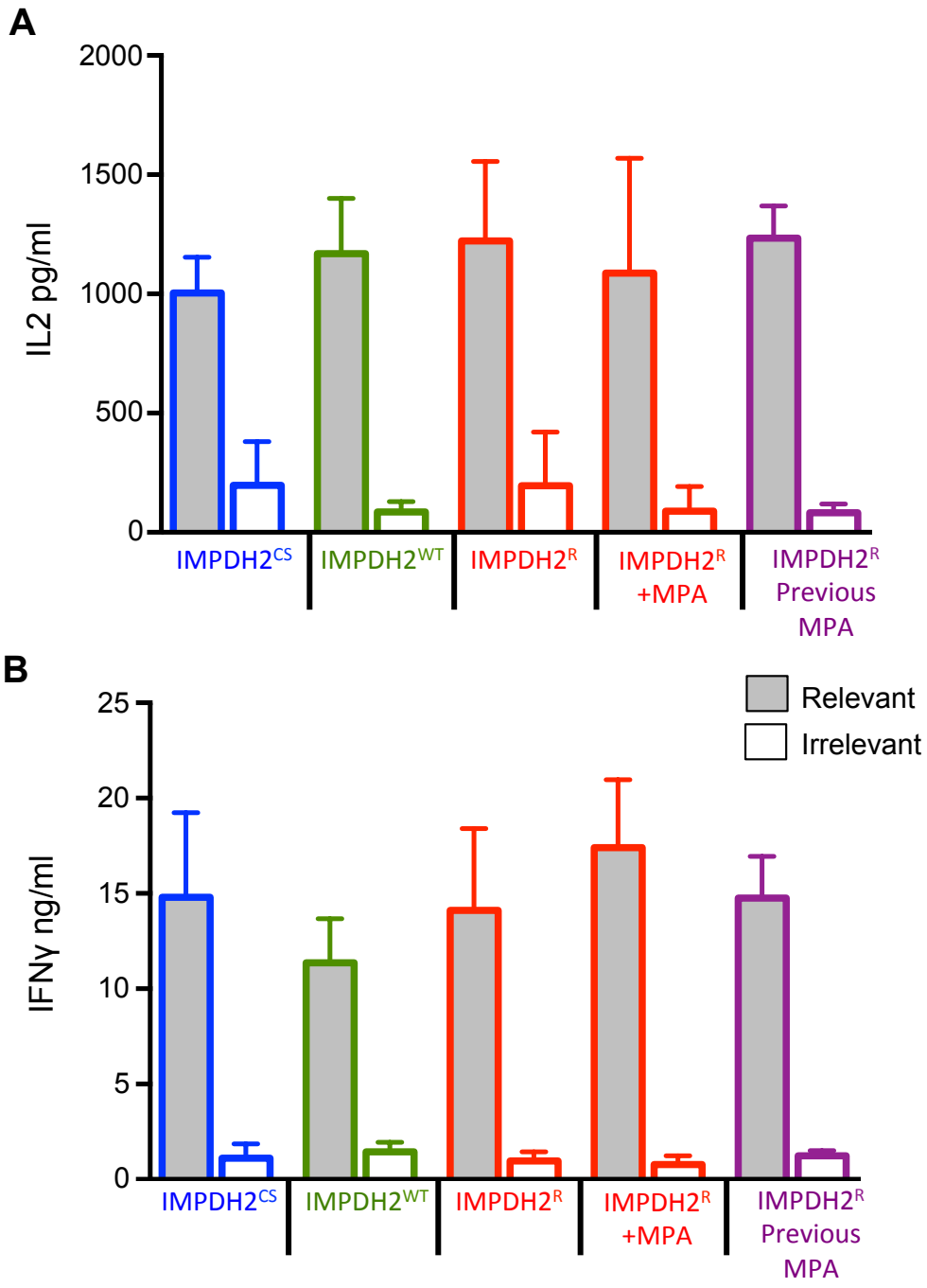

A
Baseline
GFP
"IMPDH2R Selection"
Multimer
"TCR selection"
26.3
4.03
64.3
5.45
1 Stimulation
27.0
7.0
56.5
8.58
81.2
12.7
5.78
0.38
3 Stimulations
5.94
38.1
9.52
46.5
15.2
79
2.71
3.25
30.2
69.8
0.03
0.01
No MPA
No MPA after 1st stimulation
+ 4.5µM MPA
B
Fold change –Total dual transduced cells
No MPA
MPA
5000
4000
3000
2000
1000
0
Baseline
1
2
3
C
Fold change –TCR transduced cells GFP positive %
No MPA
MPA
5
4
3
2
1
0
Baseline
1
2
3
IMPDH2CS + LMP2 TCR
IMPDH2R + LMP2 TCR (MPA for 1 stimulation)
IMPDH2WT + LMP2 TCR
IMPDH2R + LMP2 TCR

3.3.8 Retention of function post MPA exposure

To assess the ability of IMPDH2^{R} cells cultured in MPA to function, cells were stimulated overnight with peptide loaded T2 cells +/- MPA as appropriate. Supernate was collected and tested by ELISA for IL-2 (figure 3.9A) and IFN-gamma (figure 3.9B). Due to insufficient numbers of cells surviving to this time point, analysis could not be performed for IMPDH2^{CS} or IMPDH2^{WT} transduced cells exposed to MPA. For both IL-2 and IFN-gamma the level of production is similar for all groups tested. This implies that IMPDH2^{R} treated cells maintain function despite MPA exposure.

Figure 3.9 IMPDH2^{R} transduced cells selected by exposure to MPA, produce Interleukin 2 and interferon gamma in response to cognate peptide at a similar level to cells cultured in the absence of drug.

Following 3 rounds of stimulation, 5 x 10^5 dual transduced PBMC were cultured with 5 x 10^5 peptide loaded T2 cells overnight. Supernate was stored and levels of IL-2 and IFN gamma measure by ELISA as per kit instructions. Summary data from 3 independent experiments are shown for **(A)** IL2 and **(B)** IFN gamma.



3.4 Discussion

Here I have demonstrated that a murine cell line can be successfully transduced with IMPDH2^{R} and that IMPDH2^{R} transduction confers a selective advantage to these cells in the presence of MPA. The BW cell line divides rapidly and therefore was ideal to demonstrate that this selective advantage occurs due to cells overcoming both cell cycle arrest and apoptosis, known actions of MPA.

The increase in calculated IC_{50} for IMPDH2^{R} over IMPDH2^{CS} transduced BW cells was only 20 fold rather than 2400 fold previously calculated for the mutated enzyme. The previously reported increase in IC_{50} was calculated using protein purified from both wild-type and MPA-resistant neuroblastoma cell lines. In the work presented in section 3.3.1, I determined the IC_{50} by studying the effect of MPA on the growth of cells transduced with IMPDH2^{R}, IMPDH2^{WT} or IMPDH2^{CS}. Determining the IC_{50} under these conditions is likely to more accurately reflect the level of resistance potentially conferred by transduction. Within cells, variability such as the availability of substrate or the amount of mutated IMPDH2 compared to the amount of wild-type enzyme will impact the IC_{50}, effects not seen when investigating purified enzyme alone.

When investigating apoptosis, cells transduced with either IMPDH2^{WT} or IMPDH2^{R} cultured in the absence of MPA exhibited higher percentages of apoptotic cells than those transduced with IMPDH2^{CS}. During prolonged culture of sorted transduced BW cells, there was no apparent difference in the rate of proliferation of cells transduced with the different constructs. Additionally, no difference in the numbers of non-viable cells was seen.

I have also successfully transduced primary murine lymphocytes and demonstrated selection during MPA exposure of $IMPDH2^{R}$ but not $IMPDH2^{CS}$ transduced cells. To enable selection, I induced proliferation by either antigenic stimulation or cytokine. The concentration of MPA required to induce selection depended on the proliferative drive applied to the cells, with higher MPA concentrations required for cytokine than antigen driven proliferation. Selection was progressive throughout the period of MPA exposure and cells with higher copy numbers of the introduced gene appear to be preferentially selected as evidenced by higher MFI, however this did not achieve statistical significance when results from 3 independent experiments were combined. Using T cells from OT1 TCR transgenic mice provided a model where all IMPDH transduced cells are also of known antigen specificity. This does not occur when dual transducing cells with both TCR and IMPDH2 encoding retroviruses.

Overall expansion of transduced OT1 cells was greater when exposed to an irrelevant antigen rather than with SIINFEKL. This appeared to be due to increased cell death following stimulation with cognate antigen. Activation induced cell death is a well described consequence of TCR ligation (Green et al., 2003). In these experiments, the use of TCR transgenic cells and saturating levels of peptide may have contributed to this. The culture conditions included both addition of exogenous IL-2 and irradiated feeder cells, which in the absence of cognate antigen may have been sufficient for T cells to both persist and proliferate but at reduced rates compared to cells stimulated by cognate antigen. The requirement for guanosine nucleotides within these cells would have been lower and therefore MPA had less effect on these cells.

Under conditions where exogenous IL-2, IL-7 and IL-15 were added to the culture medium, there is an initial expansion of cells. However, the conditions used do not stimulate high levels of cell activation and expansion. The initial expansion phase seen is likely to relate to activation for transduction 4 days earlier with Con-A and IL7 rather than the common gamma chain cytokines used. $IMPDH2^{R}$ confers a selective advantage at all concentrations of MPA used compared to $IMPDH2^{CS}$ and $IMPDH2^{WT}$. However, $IMPDH2^{R}$ transduced cells only continue to expand in number at the higher MPA concentration used (1500nM). At a higher concentration of MPA, GTP levels in control ($IMPDH2^{CS}$ and $IMPDH2^{WT}$) cells decrease to a level that results in cell death. In contrast, $IMPDH2^{R}$ confers a significant advantage to transduced cells at this concentration. By the end of the experiments outlined in section 3.3.6, the vast majority of cells were GFP positive in the group of $IMPDH2^{R}$ transduced cells exposed to 1500nM MPA. At the lower dose of MPA only 30-50% of the cells were GFP positive reflecting the ability of untransduced cells to persist and in fact proliferate at this concentration of drug.

Using primary murine cells *in vitro* led to some difficulties. The main difficulties were limiting survival of cells post transduction and reduction in the percentage of IMPDH2 transduced cells during culture in the absence of MPA. In almost every experiment there was a decline in the percentage of transduced cells in all groups when not exposed to MPA. Despite a decline in percentage, there was still an overall increase in transduced cell number because the total cell count increased. The transduced cells were outcompeted by untransduced cells within the same culture. In order to achieve successful retroviral transduction, cells are activated prior to incubation with retroviral supernate. It is likely that the successfully transduced cell population differs from

untransduced cells i.e. cells that do not transduce may have received lower levels of initial activation. This may subsequently enable an increased rate of activation and cell division during the subsequent experimental timeframe.

In analysing my data, I have presented my findings as changes in total number of transduced cells to allow both percentage of transduced cells and expansion or contraction of cell numbers to be taken into account. In addition, I have normalised numbers to those at baseline to account for differences in initial transduction rates. This has allowed results from independent experiments to be combined.

During these experiments, I purified populations of CD8 T cells prior to activation and transduction. Following transduction, cells were cultured for 3 days with only the addition of exogenous IL-2 to T cell media. The resulting rates of cell death meant that some experiments were limited by the number of viable cells available either at baseline or during the experiment. Addition of exogenous IL-7 and IL-15 to the culture media maintained and expanded cell numbers, ensuring adequate numbers were available to initiate experiments.

I have demonstrated selection of dual-transduced TCR redirected human PBMC i.e. transduced with a known TCR (LMP2) and IMPDH2^{R}. My aim, using human cells, was to develop an experimental protocol that would mimic the *ex vivo* manipulation of cells that could subsequently be used therapeutically. The LMP2 TCR is specific for the immunodominant epitope of EBV and is an attractive TCR to use in treatment of post-transplant lymphoproliferative disorder (PTLD). While not shown here, I found similar results in one experiment in which I used a TCR specific for the NLG peptide of CMV. Despite work to optimise dual transduction, only 4-7% of cells were

successfully dual transduced at baseline. Selection for IMPDH2^{R} was almost complete after 1 round of stimulation with MPA and large numbers of dual-transduced cells could be selected after 3 rounds of stimulation. Once IMPDH2^{R} was selected, cell expansion in the absence of drug does not lead to a significant decrease in the percentage of IMPDH2^{R} transduced cells. Importantly, for therapeutic use, I have also demonstrated that cells cultured and selected in MPA retained function. While killing was not assessed, levels of cytokine production (Interleukin-2 and interferon-γ) were equivalent to those seen in cells never exposed to drug.

These data support a strategy to generate adequate numbers of TCR redirected IMPDH2^{R} transduced cells by *ex vivo* expansion of under the selective pressure of MPA for adoptive immunotherapy. I have shown that an adequate number of cells could be produced *ex vivo* and that these cells retain function in terms of cytokine production when exposed to antigen.

Chapter 4: *In vivo* selection of IMPDH2^{R} transduced cells

4.1 Introduction

I have demonstrated the protective effect of IMPDH2^R during MPA exposure *in vitro*. For adoptive immunotherapy with IMPDH2^R transduced cells to be translated for therapeutic use in humans, the protective effects seen must also be demonstrated *in vivo* at concentrations relevant to the clinical setting.

There has been significant variation in published reports regarding the dosing, diluent and route of administration of MMF *in vivo*. Different reported dosing strategies are summarized in table 4.1.

Table 4.1 Published MMF dosing from *in vivo* murine models

Route	Diluent	Dose (µg/g)	Frequency	Reference
i.p.	CMC	90	Daily	(Van Bruggen et al., 1998)
i.p.	DMSO/PBS (1:10)	30	Daily	(Mehling et al., 2000)
Oral gavage	CMC	100	Daily	(Izeradjene and Revillard, 2001)
i.p.	CMC	30 or 100	Daily	(Ramos et al., 2003)
Oral gavage	CMC	50, 100 or 150	12 hourly	(Padalko et al., 2003)
i.p.	5% Dextrose	28, 90, 120 or 200	Daily	(Shapira et al., 2004)
i.p.	PBS	100	12 hourly	(Quéméneur et al., 2004)
i.p.	5% Dextrose	40 or 80	Daily	(Koehl et al., 2007)
i.p.	5% Dextrose	50	12 hourly	
Oral	Specially formulated feed		Continuous	
i.p.	5% Dextrose	80	Daily	(Guo et al., 2010)

CMC - 0.5%Carboxymethyl cellulose /0.9% sodium chloride/0.4% Tween 80/0.9% benzylalcohol in distilled water @ 4.3 mg/ml; i.p. – intraperitoneal; DMSO – dimethyl sulfoxide; PBS – phosphate buffered saline

These studies looked at a range of *in vivo* models investigating a range of MMF effects. In particular, models of autoimmune disease e.g. SLE in (NZB x NZW F_1) (Ramos et al., 2003), have been shown to respond well to MMF. In some studies, *in vitro* effects have not been replicated *in vivo* e.g. anti-tumour and anti-angiogenesis effects (Koehl et al., 2007). There is no clear correlation between different dosing strategies and positive results.

Part of the difficulty in establishing dosing for *in vivo* models is that there remains much debate regarding what constitutes a therapeutic dose of MMF. The therapeutic range for MMF will vary depending on the indication and the use of concomitant immunosuppression.

A therapeutic range following renal transplantation was proposed in 2006 (van Gelder et al., 2006) from data that showed $AUC_{0\text{-}12h}$ under 30mg•h/L identified 87% of patients who will develop acute rejection in the first 3 months after transplant. From their data, an upper limit was set at 60 mg•h/L due to lack of additional efficacy rather than increased toxicity. Prospective trials have now shown that leukopenia and anaemia related to MMF could be reduced using dose adjustments based on the $AUC_{0\text{-}12h}$ upper limit of 60 mg•h/L(Kuypers et al., 2008). Gastrointestinal toxicity has also been shown to relate to $AUC_{0\text{-}12h}$ >60 mg•h/L confirming the benefit of the maximum $AUC_{0\text{-}12h}$ target. Trough concentrations of 1.3mg/L with CsA and 1.9mg/L with tacrolimus were proposed to ensure >80% of patients would achieve the desired $AUC_{0\text{-}12h}$.

Following liver transplantation, clinical practice suggests that a target trough level 1 mg/L is important in the first year after transplantation to minimize rejection, whereas levels should be kept below approximately 3.5 mg/L subsequently to reduce the incidence of adverse effects (Tredger et al., 2004).

But following cardiac transplantation, target MPA trough concentrations >2 mg/L when MMF is combined with tacrolimus are associated with reduced levels of rejection (Yamani et al., 2000).

A consensus report on therapeutic MMF monitoring was published in 2010 (Kuypers et al., 2010). Table 4.2 summarizes their recommendations of how best to measure MPA exposure in clinical practice. At present routine monitoring of MPA levels is not recommended. Fixed MMF dosing is used; however large variability in pharmacodynamics and pharmacokinetics (Zeng et al., 2010) may result in significant under or overdosing. In renal transplant recipients the most important determinants of variability are renal function, albumin and calcineurin inhibitor therapy (van Hest et al., 2005, Le Guellec et al., 2004, van Hest et al., 2006). A prospective trial of fixed dose versus concentration controlled MMF dosing showed no difference in treatment failure between the groups (van Gelder et al., 2008). This appeared to be due to failure of dose adjustments in patients assigned to the concentration controlled group. Approximately a third of patients on current initial MMF dosing are underexposed and at increased risk of acute rejection.

Table 4.2 Summary of MPA exposure methods

Adapted from Consensus report on therapeutic drug monitoring of mycophenolic acid in solid organ transplantation. *Clin J Am Soc Nephrol,* 5, 341-58.

Not shown due to lack of copyright permission

Figure is Table 1 p344

Consensus Report on Therapeutic Drug Monitoring of Mycophenolic Acid in Solid Organ Transplantation
Kuypers DRJ et al. Clinical Journal of the American Society of Nephrology 2010;5 341-358

A new, novel approach to monitoring has been investigated in patients treated with MMF post HSCT (Li et al., 2014). They analysed IMPDH activity to use as a marker of MPA activity. Mass spectrometric detection was used in an assay measuring the conversion of IMP to XMP. Patients were sampled at five time points after drug administration 21 days after their transplant. As seen in previous studies (Li et al., 2013), substantial variability in pharmacokinetics and pharmacodynamics was seen between patients. The IC_{50} was 3.23 mg/l total and 57.3 ng/ml unbound MPA. The IMPDH AUC was associated with CMV reactivation, non-relapse mortality and overall mortality.

Extrapolation of a therapeutic concentration (3.5g/l) to mice requires a dose of 80-100μg/g to generate similar 12-hour levels, within the range of doses shown in table 4.1. Because of the lack of consensus on dose, diluent and route of administration, I chose to administer MMF intraperitoneally to ensure each recipient received the same body weight adjusted dose. I investigated the dose and diluent during initial experiments, with dosages used covering the range used in previous reports. My models required dosing to continue for several weeks, I therefore elected to administer drug once daily to reduce the repeated need for i.p. injections.

4.2 Aims

- To investigate toxicity and actions of MMF in a steady state mouse
- To demonstrate selection of $IMPDH2^R$ transduced cells in MMF treated mice

4.3 Results

4.3.1 *In vivo* dosing and toxicity of MMF

As can be seen from the available literature, highlighted in section 4.1, there is no consensus on MMF dosing in mice, or which vehicle to use for reconstitution. I initially used the CMC buffer described in table 4.1, to allow aliquots to be stored at -20˚C for up to 5 days. On thawing, a precipitate was seen that proved difficult to resuspend even following prolonged agitation and recommended gentle heating. In contrast to this, no precipitate formed when thawing aliquots of the buffer alone. The precipitate was not seen on initial reconstitution of MMF. Some mice that received MMF in CMC buffer were noted to have extensive adhesions within the abdominal cavity. Following personal communication with a group who had published protocols using this buffer (Dra. Mª Ángeles Ramos Barrón, Hospital Universitario Marqués de Valdecilla), I discovered that they had also encountered these problems often leading to blockage of needles used to administer drug.

The manufacturer of MMF (Roche) supplies recommendations for use in humans with reconstitution in 5% Dextrose followed by administration within 3 hours. They could not provide data on drug stability outside of 3 hours, following freezing or when other diluents are used for reconstitution. I subsequently used 5% Dextrose as diluent for all further *in vivo* experiments, with administration within 1 hour of reconstitution.

To investigate the maximum tolerated dose of MMF, I initially used C57B6/J mice and administered intraperitoneal MMF at doses of 0, 200 or 400μg/g/day.

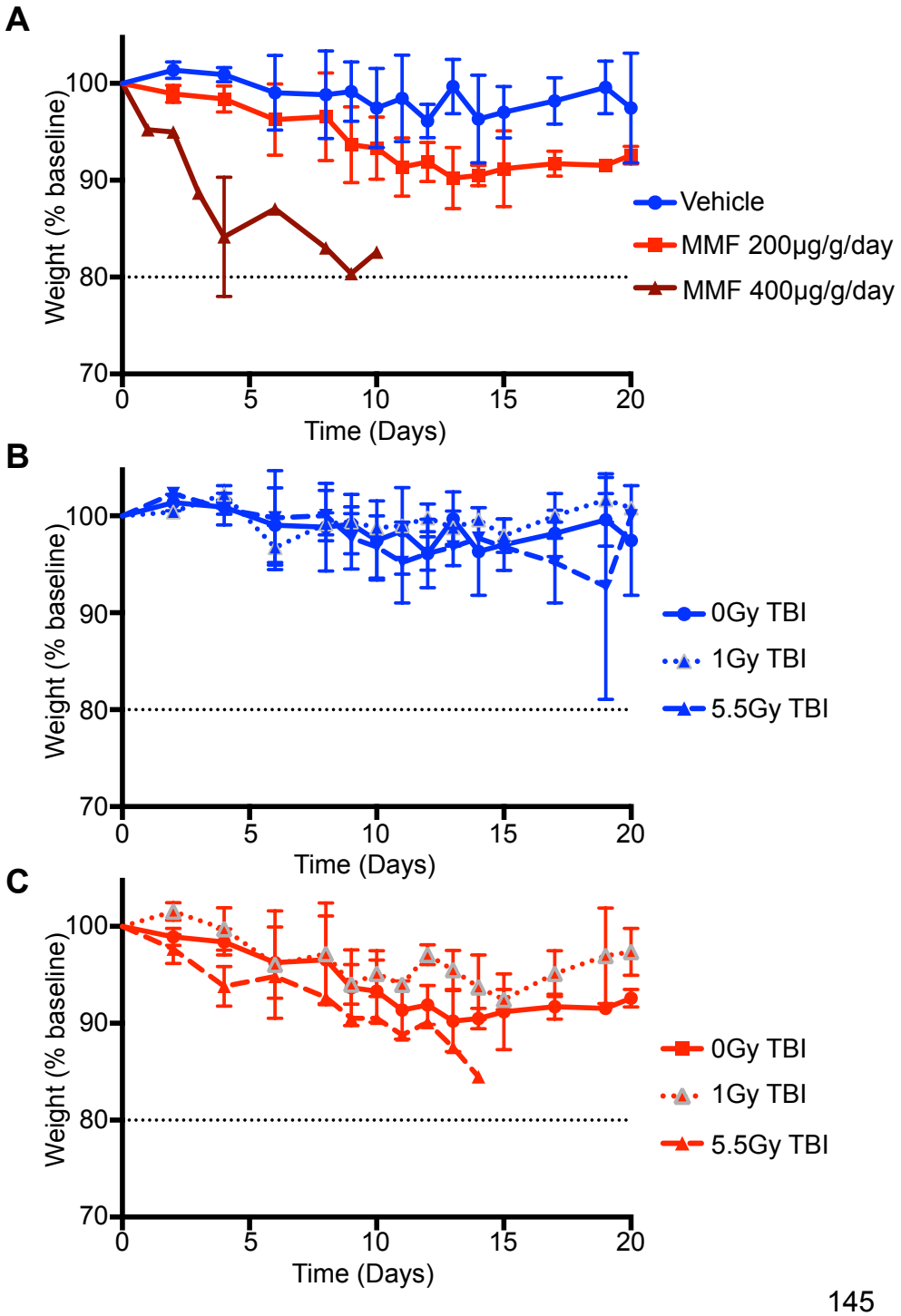
The dose of MMF administered, was adjusted to current body weight on a daily basis.

In figure 4.1, the mean weight is plotted over time as a percentage of the baseline weight for mice treated with vehicle or two doses of MMF. Mice receiving vehicle maintained their baseline weight throughout the period of MMF exposure (figure 4.1A). Mice receiving 200μg/g/day lost more weight than vehicle treated mice. On average, this was approximately 10% of body weight at 2 weeks, after which they appeared to stabilise. In contrast, mice receiving the highest dose of MMF (400μg/g/day) rapidly lost weight and all died within 11 days of initiation of treatment. Additionally, these mice appeared to develop neurological toxicity with abnormal movement patterns and behavior noted. Other than weight loss no evidence of gastrointestinal toxicity was seen.

I investigated whether the addition of lymphodepletion to MMF treatment would increase toxicity. Lymphodepletion was by total body irradiation (TBI) administered immediately prior to initiation of MMF. When combined with i.p. injections of vehicle there was little difference between weight loss in mice receiving 0, 1 or 5.5Gy of irradiation (figure 4.1B). In mice receiving the combination of 5.5Gy irradiation and MMF 200μg/g/day, significant weight loss was seen resulting in death prior to day 14. These mice also exhibited behavioural changes shortly before death including abnormal repetitive movements. Mice receiving MMF and low dose irradiation (1Gy) lost similar amounts of weight as mice receiving MMF and no irradiation.

Figure 4.1 *In vivo* MMF toxicity is manifested as dose dependent weight loss and is increased when combined with lymphodepleting irradiation

C57Bl6/J mice receiving vehicle, MMF 200µg/g/day or MMF 400µg/g/day (A) were weighed every 1-2 days. C57Bl6/J mice exposed to 0, 1 or 5.5Gy were treated with vehicle (B) or 200µg/g/day MMF (C) started on the same day as irradiation. A summary of weights plotted as a percentage of baseline over time is given. Mice were sacrificed if weight <80% of baseline. Mean and SD given.



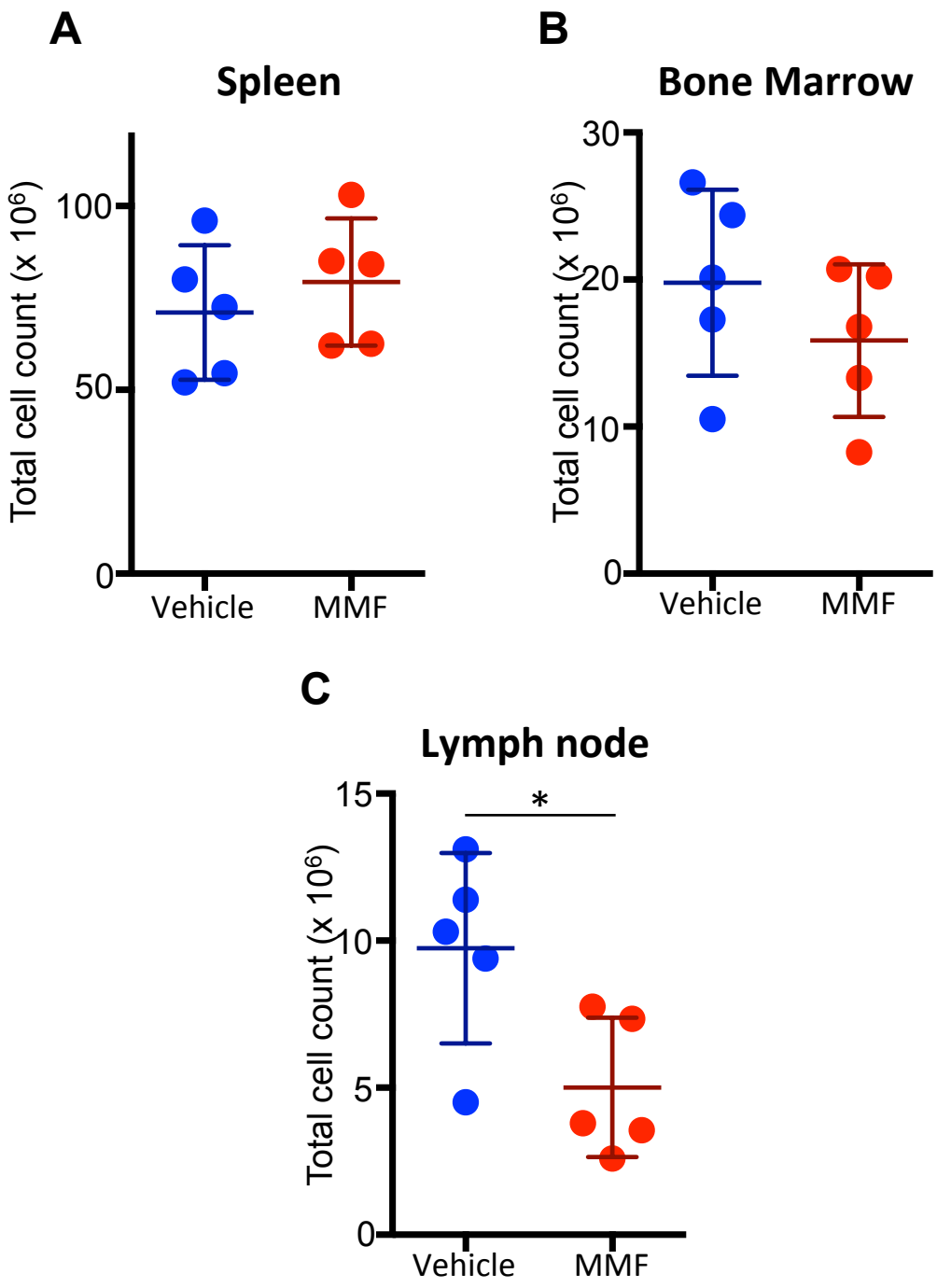
4.3.2 *In vivo* administration of MMF causes reduced lymph node cellularity in mice

I hypothesised that administration of MMF, in the absence of TBI, would result in suppression of the lymphocyte compartment. This would enable enhanced engraftment of transferred IMPDH2^{R} transduced cells by the mechanisms described in section 1.1.6 (pp. 34) for lymphodepletion. I also hypothesised that the addition of low doses of TBI would enhance the effects of MMF *in vivo* compared to the steady state.

Having identified 200µg/g/day as the maximum tolerated dose of MMF, I investigated the effects of a range of doses from 0 to 200µg/g/day on cells recovered from within the spleen, lymph nodes and bone marrow. In Figure 4.2, total cellularity within each compartment is summarised for the highest dose of MMF (200µg/g/day) compared to vehicle after 7-10 days of MMF administration. There was no difference in overall cellularity in the spleen (Mann-Whitney p=0.4127; Mean Vehicle = 71 x 10^6, MMF = 79 x 10^6) or bone marrow (Mann-Whitney p=0.4127; Mean Vehicle = 19.8 x 10^6, MMF = 15.9 x 10^6). Of note, the spleens from MMF treated mice subjectively appeared larger than those from vehicle treated mice despite no difference in cellularity. There was a significant difference in total lymph node cellularity between mice treated with vehicle and MMF (Mann-Whitney p=0.0317; Mean Vehicle= 9.74 x 10^6, MMF= 5.012 x 10^6). At dissection, the lymph nodes of mice in the MMF group were notably smaller than those in the vehicle group and were at times very difficult to locate.

Figure 4.2 MMF causes reduced lymph node cellularity.

Mice given vehicle or MMF 200µg/g/day were sacrificed at 7-10 days and the cellularity of spleen (A), bone marrow (B) and lymph node (C) assessed. Mean and SD are plotted for the total count from each organ.



4.3.3 *In vivo* administration of MMF reduces B220 cell numbers but not CD4 or CD8 T cells

In addition to the clear reduction in lymph node cellularity, a repeated finding from these experiments was a reduction in the percentage of cells with the size and granularity of lymphocytes based on flow cytometric forward and side scatter. An experiment using a range of MMF doses from 0-200µg/g/day (figure 4.3 A & B) showed a dose-dependent reduction in both the percentage and absolute number of cells within this gate when MMF doses greater than 100µg/g/day were administered. Further investigation showed that the reduction in cells within this gate was due to reduction in B220 positive cells (Figure 4.3 C & D). The percentages and absolute numbers of CD8 and CD4 T cells were not reduced by 200µg/g/day in spleen (Figure 4.4 A & B) or bone marrow (Figure 4.4 C & D). In the lymph nodes, the percentages of CD4 and CD8 T cells were unchanged (Figure 4.4E) but absolute numbers were reduced (Figure 4.4F) reflecting the reduction in absolute cellularity (Mann-Whitney CD8 p= 0.0079; Mean vehicle = 3.4 x 10^6, MMF=1.1 x 10^6; CD4 p= 0.0079; Mean vehicle = 4.5 x 10^6, MMF= 1.4 x 10^6)

Figure 4.3 MMF causes a dose-dependent reduction in lymphocytes within the bone marrow due to reduction in B220 positive B cells.

Mice given vehicle or a range of MMF doses were sacrificed after 7 days and bone marrow harvested. Cells in a lymphocyte gate (determined by forward and side scatter and back-gating of CD4 and CD8) are given as a percentage of live cells (A) and as absolute number (B). Mice receiving MMF 200µg/g/day were sacrificed at 7-10 days. Cells were analysed by FACS using the B cell marker B220. The percentage of live cells (C) and absolute number (D) with mean and standard deviation are plotted for bone marrow.

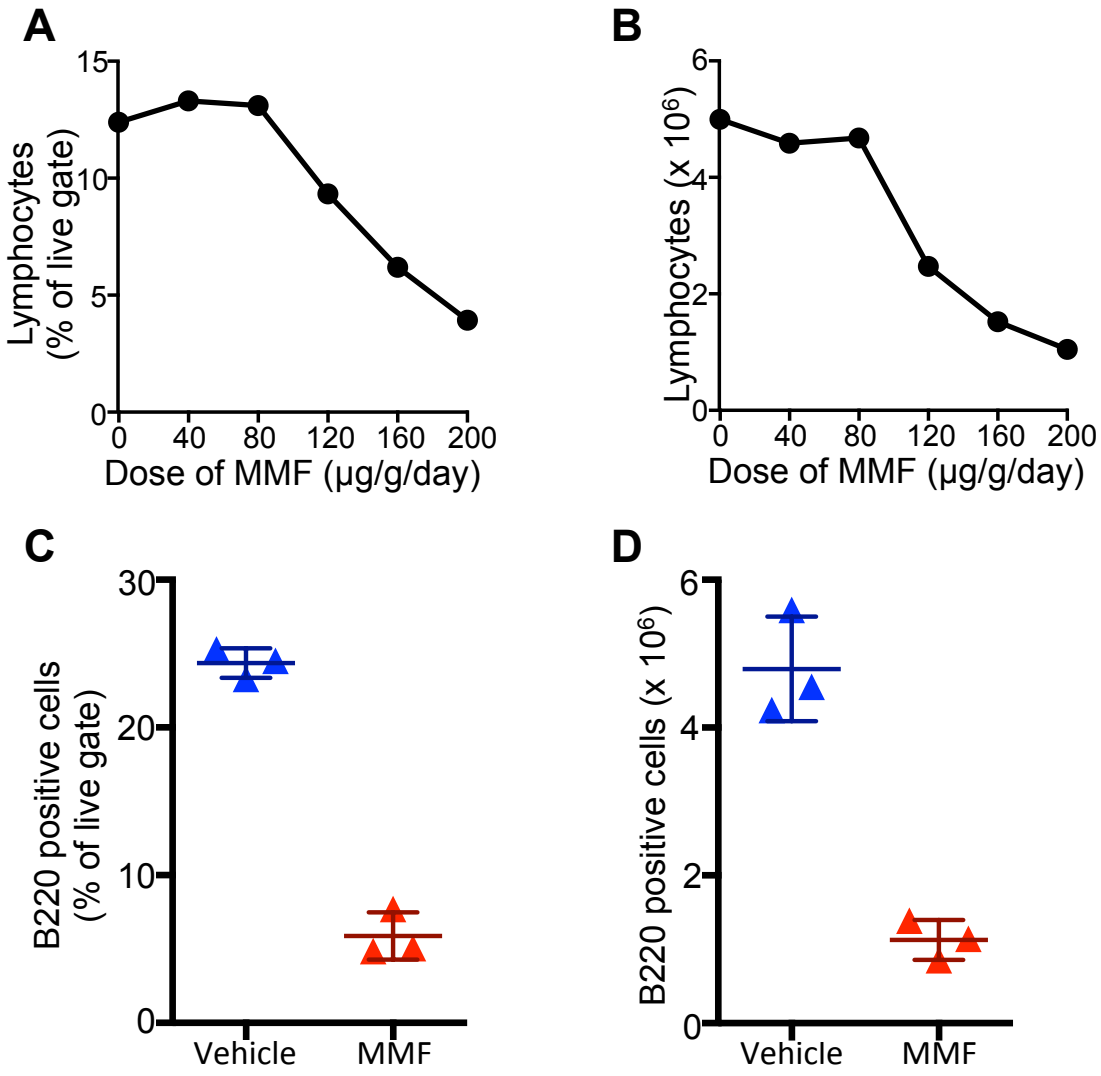
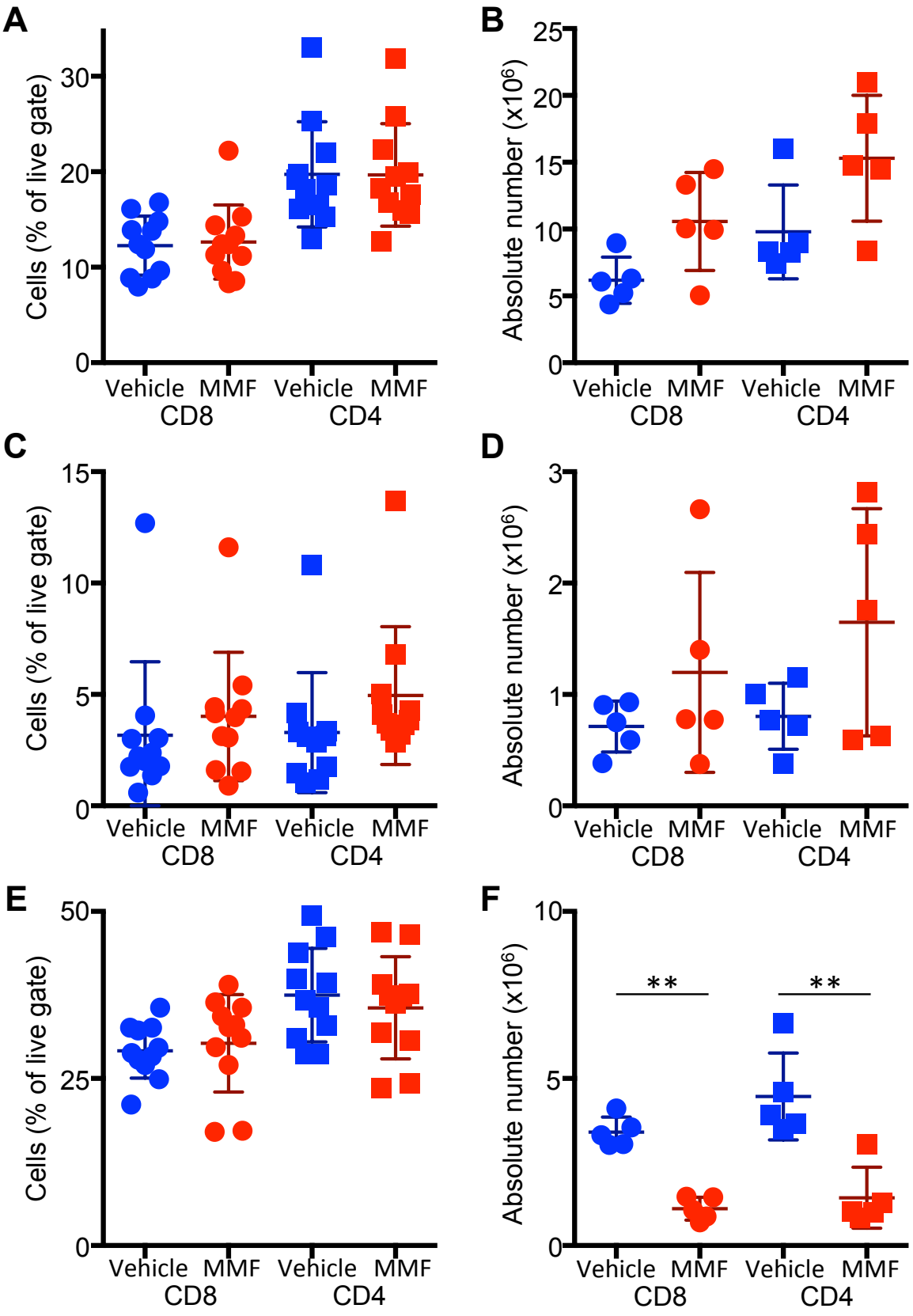


Figure 4.4 At the maximum tolerated dose of MMF, there is no change in CD4 or CD8 cells percentage but absolute numbers are decreased in lymph nodes.

Mice were treated with vehicle or MMF 200µg/g/day for 7-21 days. Spleen, left hind limb and lymph nodes (axillary, brachial and inguinal) were harvested and single cell suspensions counted and stained for FACS analysis. CD8 and CD4 positive cells are presented as percentage of live gate and absolute count for Spleen (A and B), Bone Marrow (C and D) and lymph node (E and F). Results from 5 independent experiments, mean and standard deviation are plotted.

Figure 4.4


A
Cells (% of live gate)
30
20
10
0
Vehicle MMF Vehicle MMF
CD8 CD4
B
Absolute number (x10⁶)
25
20
15
10
5
0
Vehicle MMF Vehicle MMF
CD8 CD4
C
Cells (% of live gate)
15
10
5
0
Vehicle MMF Vehicle MMF
CD8 CD4
D
Absolute number (x10⁶)
3
2
1
0
Vehicle MMF Vehicle MMF
CD8 CD4
E
Cells (% of live gate)
50
25
0
Vehicle MMF Vehicle MMF
CD8 CD4
F
Absolute number (x10⁶)
10
5
0
**
**
Vehicle MMF Vehicle MMF
CD8 CD4

In Vivo Selection of IMPDH2^{R}

Having seen minimal effect of MMF on CD4 and CD8, I hypothesised that homeostatic proliferation of lymphocytes is at a rate insufficient for MMF to cause suppression. In order to test the *in vivo* effects of MMF on cells stimulated to proliferate, I designed the experimental protocol outlined in figure 4.5.

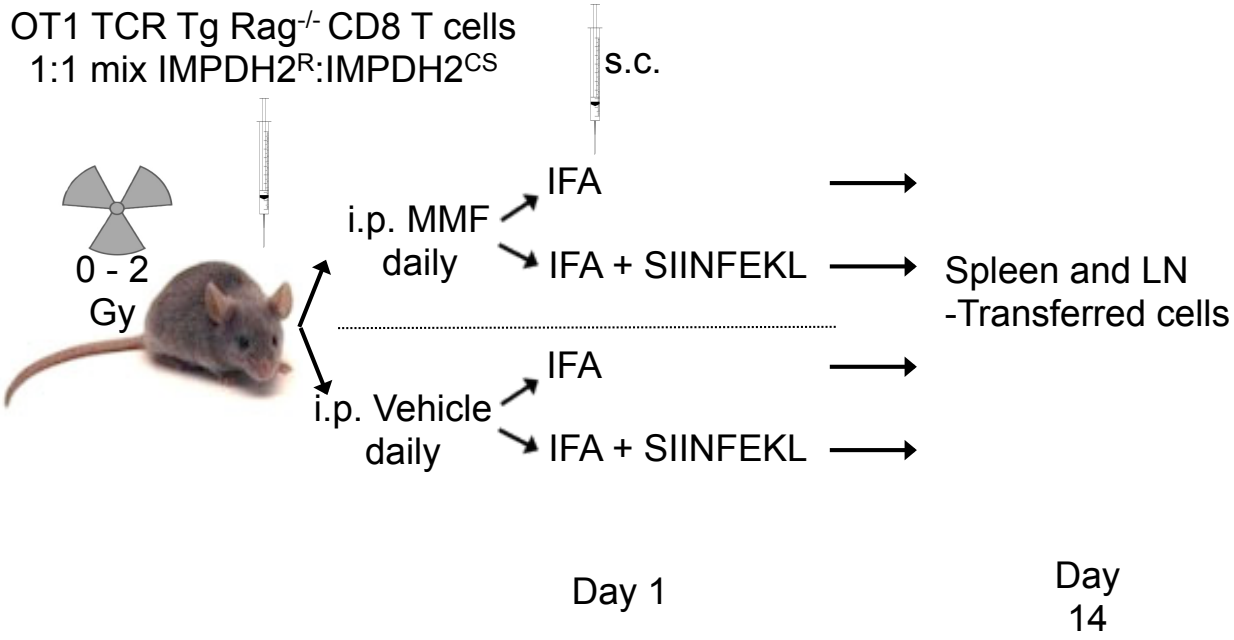
In these experiments, I stimulated proliferation by two mechanisms;

1. Lymphopenia
2. Cognate antigen

Lymphopenia-induced proliferation resulted from TBI. Low doses were used to avoid the toxicity seen in pilot experiments when TBI and MMF were given in combination. Stimulation with cognate antigen within this model ensured that transferred but not endogenous cells would be stimulated. I used a model antigen system and selected donor mice transgenic for the OT1 TCR, also used *in vitro* see section 3.3.5, stimulated by administration of the cognate OVA peptide (SIINFEKL) given with adjuvant.

Figure 4.5 Overview of protocol to investigate *in vivo* selection of IMPDH2^{R} transduced cells

C57Bl6/J mice were exposed to low doses of irradiation (0, 1.5 or 2Gy) and MMF 0, 100 or 200µg/g/day started. A total of 1 x 10^6 transduced OT1 Rag$^{-/-}$ cells were transferred intravenously 4-6 hours later. The transferred cells contained a 1:1 mix of IMPDH2^{CS} and IMPDH2^{R} transduced cells. Both donor types could be differentiated from each other and the recipient using the congenic markers Thy1.1/1.2 and CD45.1/45.2. Mice received either Incomplete Freud's adjuvant (IFA) or an emulsion of SIINFEKL in IFA on day 1 by subcutaneous injection at the base of the tail. Weight was monitored during 14 days of MMF administration. Mice were sacrificed at day 14 with spleen and draining lymph nodes (inguinal and para-aortic) harvested for FACS analysis.



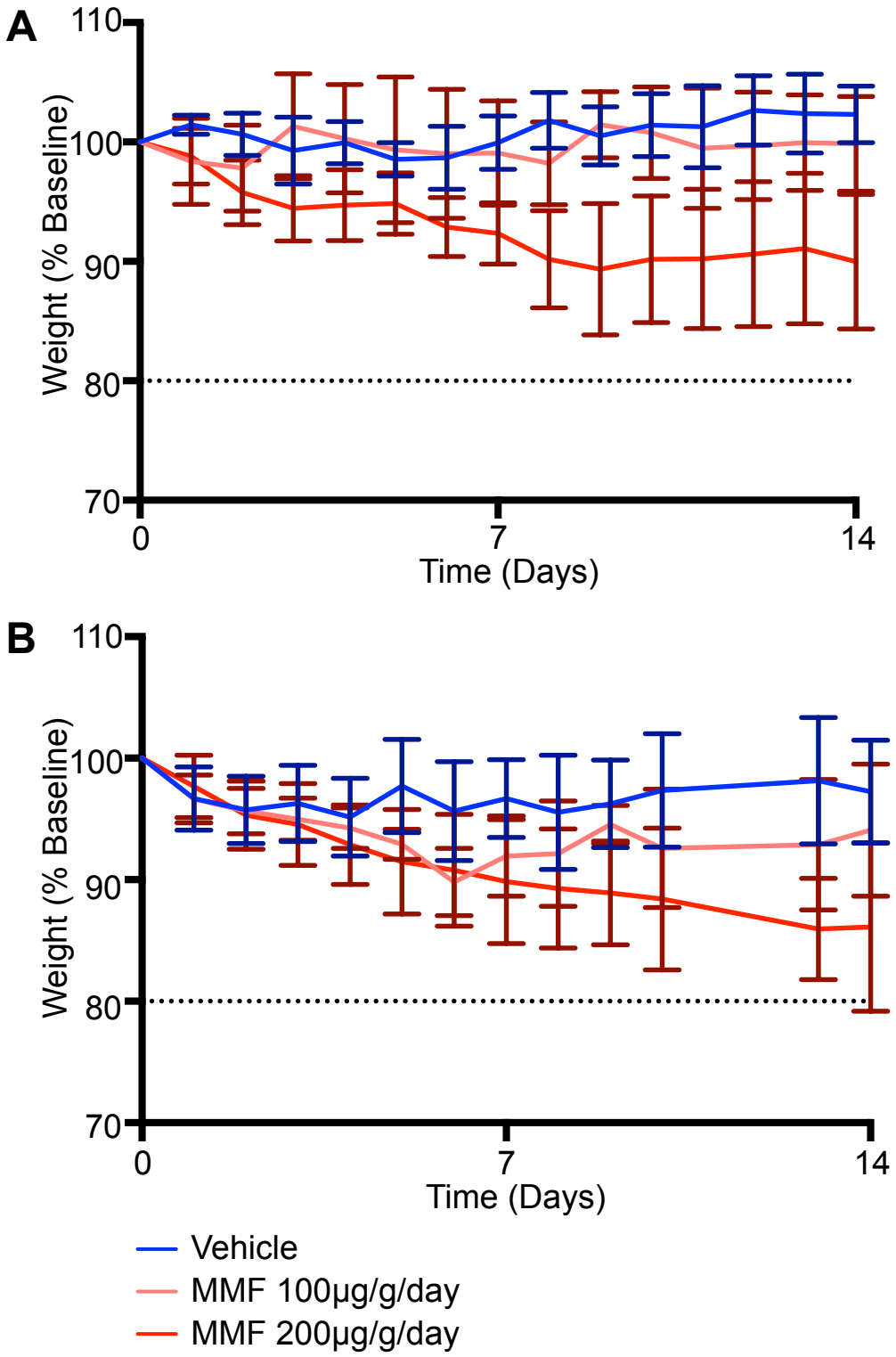
4.3.4 Development of a model of *in vivo* selection

As seen in figure 4.6, there was MMF dose-dependent weight loss in both TBI and No TBI groups. In the absence of TBI (figure 4.6A), mice receiving vehicle or 100μg/g/day MMF maintained body weight throughout the experiment. Mice receiving MMF 200μg/g/day progressively lost weight until day 10 after which it stabilised at ~90% of baseline. Weight loss in all groups was greater following TBI. Vehicle treated mice lost <5% of baseline weight before stabilising after 4-5 days. Mice receiving MMF lost ~10% of weight during the first week of the experiment with mice receiving 200μg/g/day continuing to lose weight up until the end of the experiment. In the higher-dose group several mice were found dead or required sacrifice before the end of the experiment due to weight loss. In the 100μg/g/day group, weight stabilised after 7 days and gradually increased during the second half of the experiment.

I hypothesised that when cells were transferred under conditions to stimulate proliferation, IMPDH2^{R} transduced cells would be preferentially selected over IMPDH2^{CS} transduced cells. In order to detect whether selection had occurred, I utilised donor mice of two different types that could be distinguished from each other and recipient mice by the congenic markers Thy1.1 and Thy1.2 and CD45.1 and CD45.2.

Figure 4.6 Weight loss is increased at higher MMF doses and when MMF and TBI are combined

The weights of mice undergoing the experimental protocol outlined in figure 4.4 were taken every 1-2 days. Weight is given as a percentage of baseline weight and shown over time for recipients of no TBI (A) and TBI (B).



Example FACS plots of splenocyte samples from mice receiving 2Gy TBI and 100µg/g/day of MMF or vehicle are shown in figure 4.7. Samples were initially gated for CD8 positive cells and then transferred cells (Thy 1.2 positive). Across repeated experiments, the transduction efficiency (GFP positivity) was between 30 and 70% of cells at transfer. The ratio between $IMPDH2^{R}$ and $IMPDH2^{CS}$ was set on transduced (GFP positive) cells only. In the example shown, just under 50% of GFP positive cells are also CD45.1 positive ($IMPDH2^{R}$) to give a baseline ratio of 0.9. In each of the multiple experiments performed, the baseline ratio at transfer was close to but not exactly 1. To ensure that the different congenic forms of CD45 did not influence results, the donor type transduced with $IMPDH2^{R}$ was switched in different experiments.

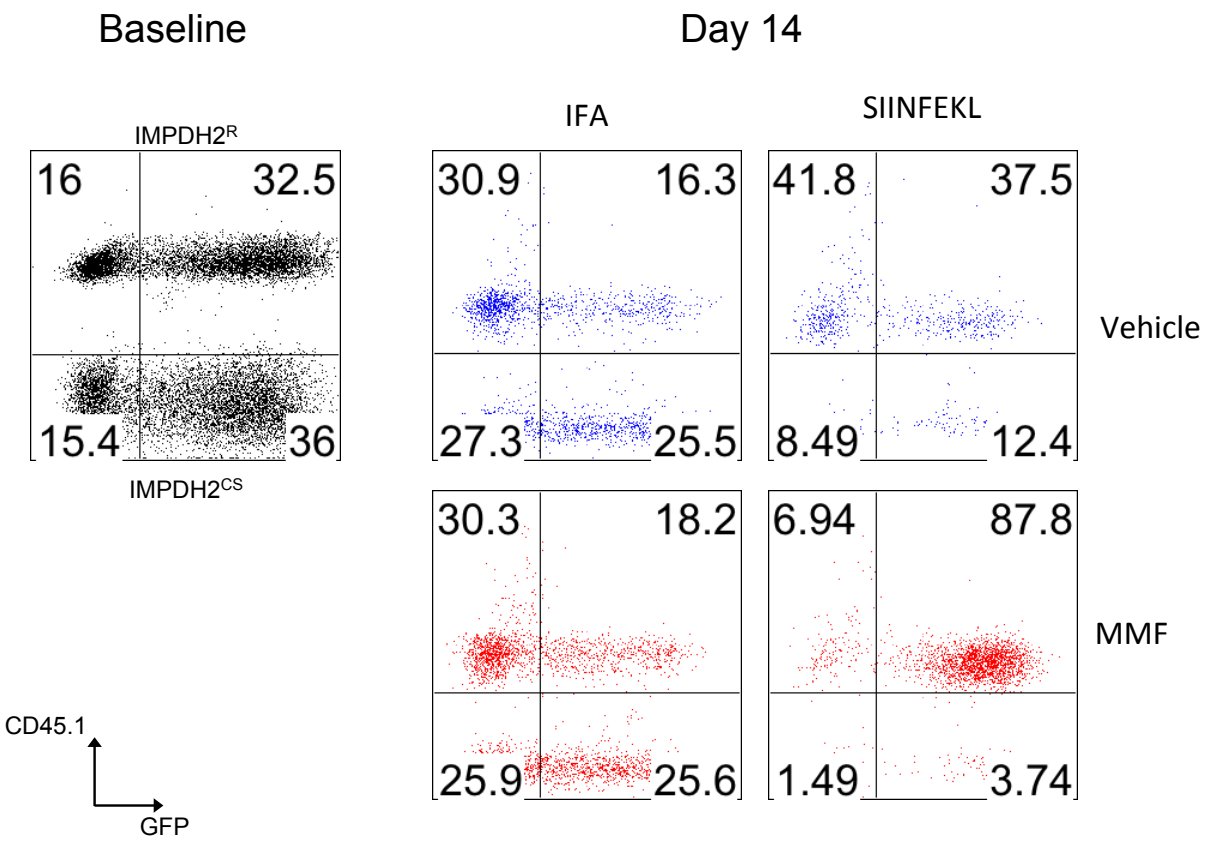
After 14 days, vehicle treated mice given IFA alone do not exhibit selection for $IMPDH2^{R}$ transduced cells compared to $IMPDH2^{CS}$ (ratio 0.639) but with antigen and vehicle there is selection seen (ratio 3.02). MMF treatment resulted in strong selection of CD45.1 positive GFP positive cells in the presence of antigen (ratio 23.5) but not in the absence of antigen (ratio 0.7109). Of note, in the plot from a mouse receiving antigen and vehicle there is a shift in the ratio for both transduced and untransduced cells. This was not replicated in other experiments.

To summarise data from multiple experiments, the calculated ratio was corrected for the baseline ratio for that experiment. During initial experiments, spleen, lymph node and bone marrow samples were collected. The number of transferred cells in bone marrow was so low that data is not included for analysis. The results shown in figures 4.8-4.10 are combined from 9 independent experiments using a total of 12 different injection mixes.

Recipient mice fell into a total of 12 groups, 6 with TBI and 6 without. With and without TBI groups received 0, 100 or 200 μg/g/day MMF and received either antigen or adjuvant alone. Mice in each of these groups had data recorded to demonstrate selection in spleen and lymph node samples. Because of the number of recipient mice required, there were only 3 available in each no TBI group. The data from both doses of MMF are also presented in combination in these groups.

Figure 4.7 IMPDH2^{R} cells are selected over IMPDH2^{CS} cells during *in vivo* MMF administration

Example plots are shown of selection in mice receiving 2Gy irradiation and an MMF dose of 100µg/g/day. Samples of splenocytes are pre-gated on CD8 and Thy1.2 (transferred cells). Plots show CD45.1 against GFP. CD45.1 positive cells were transduced with IMPDH2^{R} while IMPDH2^{CS} transduced were CD45.1 negative. Plots are taken at baseline of the injection mix and at day 14 from vehicle treated or MMF treated mice and are shown both with and without antigen.



4.3.5 Selection of IMPDH2^{R} requires both antigenic stimulation and high dose MMF In non-irradiated mice

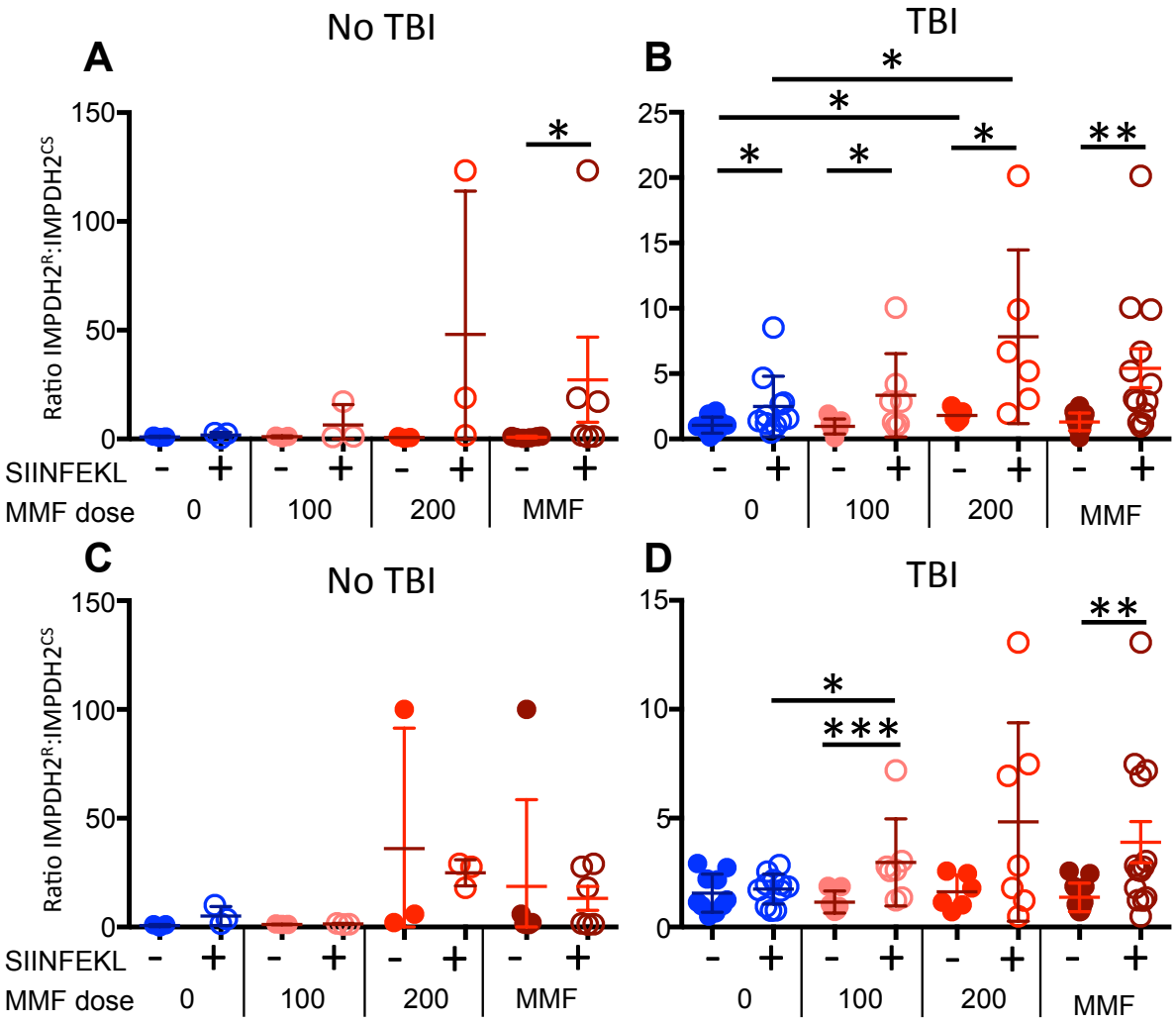
Summary data for mice not receiving lymphodepleting irradiation is shown for the spleen (figure 4.8A) and draining lymph nodes (figure 4.8C). Selection is summarised as a ratio of IMPDH2^{R}:IMPDH2^{CS} with a larger number signifying selection of resistant cells. Under conditions where there is no prior lymphodepletion, there is no MMF induced selection in the absence of antigen. Of note there is one outlying result from the group receiving IFA alone and 200µg/g/day of MMF where no IMPDH2^{CS} transduced cells could be detected.

When proliferation of transferred cells is induced by cognate antigen there is a trend towards selection at 200µg/g/day MMF (Mann-Whitney IFA v IFA+OVA MMF 200µg/g/day p=0.1; Mean IFA=0.655, IFA+OVA=48) however as only 3 mice were available for each group significance could not be reached in this experiment. If both MMF doses are combined a significant difference is seen (Mann-Whitney MMF IFA v IFA+OVA p=0.0411; 0.846 v 27.22).

In draining lymph nodes from these mice exposed to cognate antigen, a similar pattern can be seen but this does not reach significance even when MMF groups are combined and outliers removed. There is a trend towards selection in the lymph node without MMF (Mann-Whitney Vehicle & IFA v IFA+OVA p=0.1; 0.692 v 5.176) and trends towards greater selection with higher doses of MMF (Mann-Whitney IFA+OVA 0 v 100µg/g/day and 0 v 200µg/g/day both p=0.1; Mean: Vehicle=5.176, 100µg/g/day=1.076, 200µg/g/day=24.06).

Figure 4.8 Ratio of IMPDH2^{R}:IMPDH2^{CS} transduced cells

Mice treated under the protocol outlined in figure 4.4 were sacrificed 14 days after adoptive transfer and samples of cells from spleen and lymph nodes analysed for transferred cells. The ratio of IMPDH2^{R}:IMPDH2^{CS} transduced cells is given, normalised to the input ratio. Results are combined from 9 independent experiments. Data from mice receiving no TBI are summarised for spleen (A), and lymph node (C) while mice receiving TBI are summarised for spleen (B) and lymph node (D). Mean and standard deviation are plotted. IFA alone = closed, SIINFEKL + IFA = Open, MMF dose in µg/g/day



4.3.6 Following TBI, selection of IMPDH2^R is driven by antigenic stimulation and increased by MMF administration within the spleen

Summary data for spleen (figure 4.8B) and draining lymph nodes (figure 4.8D) are shown for mice receiving low dose TBI. Following lymphodepletion, cognate antigen can drive selection of IMPDH2^R transduced cells. This was independent of but enhanced by treatment with MMF. In the spleen significant selection was seen in vehicle treated mice when transferred cells were stimulated with cognate antigen (Mann-Whitney IFA v IFA+OVA Vehicle p=0.0356; Mean IFA=1.061, IFA+OVA= 2.492). This was also the case at both 100µg/g/day (Mann-Whitney IFA v IFA+OVA 100µg/g/day MMF p=0.0262; Mean IFA=0.9609, IFA+OVA= 3.341) and 200µg/g/day of MMF (Mann-Whitney IFA v IFA+OVA 200µg/g/day MMF p=0.0173; Mean IFA=1.794, IFA+OVA= 7.823). MMF at the higher dose exhibited greater selection of IMPDH2^R transduced cells than vehicle with or without antigen (Mann-Whitney MMF 0 v 200µg/g/day IFA alone p=0.04; IFA+OVA p=0.0103).

Compared to the results from spleen samples, selection in lymph node samples from mice receiving TBI did not exhibit clear dose-response and the effect of antigen was less apparent. At the lower dose of MMF there was significant selection with antigen compared to no antigen (Mann-Whitney IFA v IFA+OVA 100µg/g/day MMF p=0.007; Mean IFA=1.062, IFA+OVA= 3.403). At the higher dose of MMF the effect of antigen was non-significant (Mann-Whitney IFA v IFA+OVA 100µg/g/day MMF p=0.0519; Mean IFA=1.648, IFA+OVA= 5.578). Antigen was also not a significant driver of selection in the absence of MMF (Mann-Whitney IFA v IFA+OVA Vehicle p=0.1419; Mean IFA=1.407, IFA+OVA= 1.916). Neither dose of MMF significantly increased selection of IMPDH2^R transduced cells over vehicle treated mice (Mann-

Whitney IFA+OVA 0 v 100µg/g/day MMF p=0.0853, 0 v 200µg/g/day MMF p=0.0727; Mean Vehicle=1.916, 100µg/g/day MMF=3.403, Mean 200µg/g/day MMF=5.578).

4.3.7 Selection of IMPDH2^{R} within the activated (CD44^{high}) compartment requires lymphodepletion and is driven by both antigen and MMF

To investigate whether selection was increased in activated T cells, I gated for CD44^{high} transferred T cells prior to calculating a ratio of transduced cells. These results are shown in figure 4.9 with absolute numbers of IMPDH2^{R} cells shown in figure 4.10. In the absence of TBI (Figure 4.9A and C) there is no significant selection seen even when MMF doses are combined. In contrast, following TBI there is significant selection for IMPDH2^{R} transduced cells in both the spleen and lymph nodes.

Within the spleen, selection is driven by antigen (Figure 4.9B) in the presence of MMF (Mann-Whitney test IFA v IFA + SIINFEKL 100µg/g/day MMF p=0.0379, 200µg/g/day MMF p=0.0101; Means 100µg/g/day IFA = 1.436, IFA + SIINFEKL 4.156, 200µg/g/day IFA = 1.9, IFA + SIINFEKL 9.785) but a non-significant trend is seen in the absence of MMF (Mann-Whitney test IFA v IFA + SIINFEKL Vehicle p=0.0558; Means IFA = 1.229, IFA + SIINFEKL = 2.008).

This result is mirrored in the lymph nodes (Figure 4.9D; Mann-Whitney test IFA v IFA + SIINFEKL Vehicle p=0.059, 100µg/g/day MMF p=0.0169, 200µg/g/day MMF p=0.014; Means Vehicle IFA = 1.229, IFA + SIINFEKL = 2.008, 100µg/g/day IFA = 1.207, IFA + SIINFEKL 3.264, 200µg/g/day IFA = 1.469, IFA + SIINFEKL 5.872).

MMF increases the level of selection over vehicle in both spleen and lymph node following antigen exposure. Within the spleen this is only at the higher dose of MMF (Mann-Whitney test MMF Vehicle v 100µg/g/day p=0.1788, Vehicle v 200µg/g/day p=0.0019). In the lymph node it is increased at both doses (Mann-Whitney test MMF Vehicle v 100µg/g/day p=0.0268, Vehicle v 200µg/g/day p=0.0114).

There are significantly increased numbers of activated IMPDH2^{R} transduced cells seen following addition of antigen to MMF in the absence of lymphodepletion (figure 4.10A and C). In general, the transduced cell numbers were greater following lymphodepletion (figure 4.10B and D). Within the spleen, IMPDH2^{R} numbers were significantly higher with than without MMF.

In summary in the absence of lymphodepletion, antigen driven proliferation and MMF are both required to select resistant cells. Following lymphodepletion, IMPDH2^{R} cells are selected with antigen alone and this effect is even greater when MMF is given. This also results in increased numbers of activated resistance transduced cells following lymphodepletion, antigen and MMF than without.

Figure 4.9 Ratio of Activated (CD44^{high}) IMPDH2^{R}:IMPDH2^{CS} transduced cells

Mice treated under the protocol outlined in figure 4.4 were sacrificed 14 days after adoptive transfer and samples of cells from spleen and lymph nodes analysed for transferred cells. The ratio of IMPDH2^{R}:IMPDH2^{CS} transduced cells is given, normalised to the input ratio. Results are combined from 9 independent experiments. Data from mice receiving no TBI are summarised for spleen (A), and lymph node (C) while mice receiving TBI are summarised for spleen (B) and lymph node (D). Mean and standard deviation are plotted. IFA alone = closed, SIINFEKL + IFA = Open, MMF dose in µg/g/day. Results significant by Mann-Whitney test are shown.

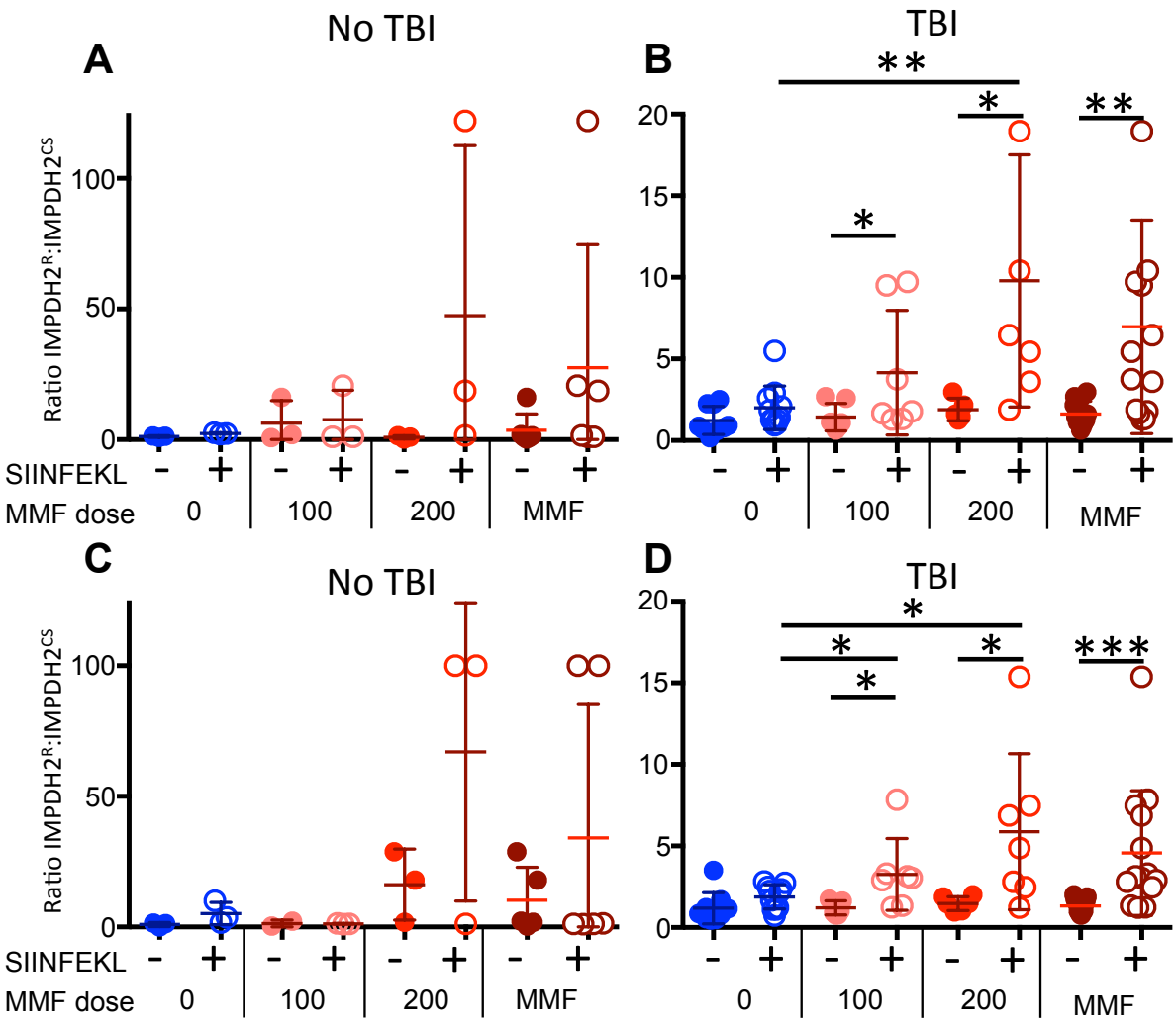
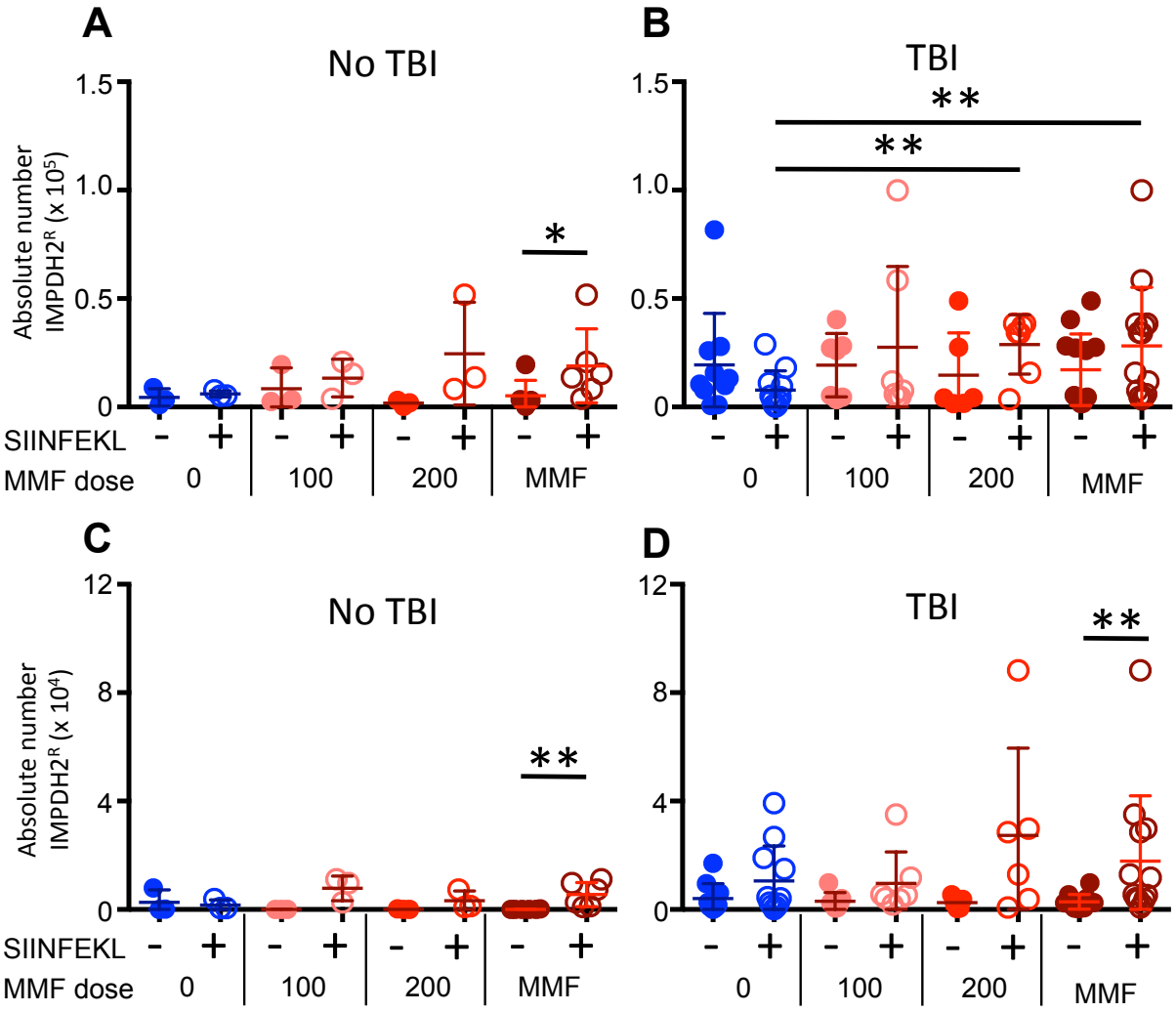


Figure 4.10 Absolute numbers of CD44^{high} IMPDH2^{R} transduced cells

Mice treated under the protocol outlined in figure 4.4 were sacrificed 14 days after adoptive transfer and samples of cells from spleen and lymph nodes analysed for transferred cells. Results are combined from 9 independent experiments. The absolute number of CD44^{high} IMPDH2^{R} cells is shown. Summary data from mice receiving no TBI spleen (A), and lymph node (C) and TBI spleen (B) and lymph node (D) are shown. Mean and standard deviation are plotted. IFA alone = closed, SIINFEKL + IFA = Open, MMF dose in µg/g/day. The two columns furthest to the right of each plot contain both MMF doses combined. Results significant by Mann-Whitney test are shown.



4.4 Discussion

The data reported in this chapter, show that daily i.p. injection of MMF in C57Bl6 mice results in limiting toxicity above 200µg/g/day. Toxicity is increased by the delivery of lymphodepleting irradiation. Mice lose weight and at higher doses of drug exhibit abnormal behaviour that may reflect neurological toxicity.

A limitation of *in vivo* use of MMF is the half-life, approximately 12-16 hours. Administration in humans is therefore 2 or 3 times daily to maintain minimum trough levels. In the experiments shown in this chapter, MMF was administered for up to 21 days. Once daily dosing of MMF was used in order to limit the number of i.p. injections required for each mouse. Once daily dosing may result in several hours each day during which drug concentrations are sub-therapeutic prior to the next dose. During this window, the inhibitor effect of MMF may be reduced to a point where sufficient levels of GMP can be produced to avoid apoptosis and initiate cell cycling. Despite this potential problem with dosing, protocols using MMF once daily *in vivo* have been reported that result in successful treatment of autoimmune diseases and in some cases suppression of tumour growth.

The *in vivo* models of autoimmunity in which MMF successfully abrogated disease are primarily antibody mediated. This is reflected in my data that show that B220 positive but not CD4 and CD8 positive cells are significantly suppressed by MMF. This represents a B cell population. This could be considered surprising as B lymphocytes have been shown to possess over twice the activity of the salvage pathway enzyme HGPRT compared to T lymphocytes (Davis and Rambotti, 1982). The action of this enzyme should

counteract the action of MMF by 'recycling' guanosine nucleotides in an IMPDH independent fashion. The presence of HGPRT in B lymphocytes was exploited to select hybridomas formed between B cells and myeloma cells to produce monoclonal antibodies (Kohler and Milstein, 1975). Patients with Lesch-Nyhan syndrome (HGPRT deficiency) have apparently normal B cells, however they show a reduced response following mitogen stimulation (Gelfand et al., 1978). In humans with SLE treated with MMF, there is no difference in the frequencies or absolute numbers of peripheral blood B cells compared to no immunosuppression (Eickenberg et al., 2012). B cells from patients treated with MMF were less able to proliferate and form plasmablasts in response to CpG or anti-CD40 + IL-21. In patients receiving immunosuppression following heart transplantation, there was a significant decline in B cells of patient on MMF compared to azathioprine treated patients and a healthy control group (Weigel et al., 2002). The patients all also received ciclosporin and prednisolone. The decline in B cell numbers started 3 months after starting MMF and by 12 months levels were nearly 50% of the other groups. This was associated with a reduction in CD38 a marker of activated B cells.

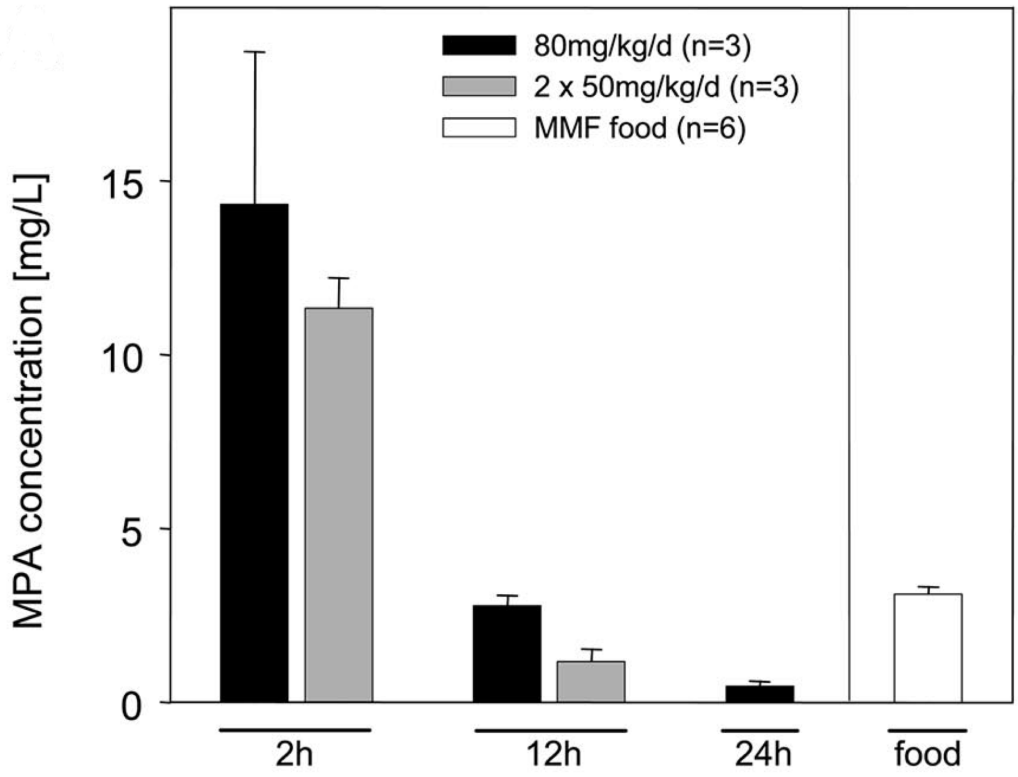
A study looking at responses to sheep red blood cell vaccination in Balb/c mice treated with MMF, showed a reduction in B-1a cells within the spleen (but not B-1b or B2 cells) at 2 week but after initial reduction in IgM levels at day 5 they were normal by day 14 (Salinas-Carmona et al., 2009). In C3H mice, MMF was shown to reduce numbers of CD19+ B cells within the spleen (Padalko et al., 2003). From the available data it appears that MMF has different effects in different body organs and there may be differences between species and depending on the underlying condition. Data from several sources supports my finding of a B lymphocyte depleting effect for MMF.

Not all published reports of MMF use *in vivo* have been successful. Koehl *et al* were unable to replicate anti-tumour and anti-angiogenic effects seen *in vitro* when MMF was administered once daily *in vivo.* They therefore investigated drug concentrations following dosing (Koehl et al., 2007). Figure 4.11 reproduced from Koehl *et al*'s work shows MPA levels in the blood of mice on 3 dosing strategies; 80mg/kg once daily, 50mg/kg twice daily and specially produced feed containing MMF. Levels achieved 2 hours after 80mg/kg MMF i.p. were within the range considered therapeutic in humans however after 12 and 24 hours the levels were subtherapeutic. The lower initial dose (50mg/kg) produced lower peak levels and was subtherapeutic prior to the 12-hour dose. The twice-daily regimen did not replicate the *in vitro* anti-tumour effects despite a shorter duration of sub-therapeutic drug concentrations each day. The alternative strategy where drug was administered continuously in feed resulted in low levels of drug throughout (3mg/l) and again did not replicate *in vitro* effects. The group repeated their *in vitro* experiments, but only exposed cells to MPA for 2 hours twice a day rather than the continuous exposure used initially. Under these conditions the *in vitro* effects were lost. Their data suggest that once daily dosing *in vivo* is able to produce therapeutic peak drug levels but as predicted levels were sub-therapeutic for long periods of the day.

Figure 4.11 MPA levels in murine blood measured after various delivery strategies.

Results show that although one or two bolus doses of MMF per day produce a relatively high early peak in MPA, levels decrease to subtherapeutic values by 24 hr. Using MMF-containing food, levels are maintained in the therapeutic range. Results shown are the mean ± SEM from multiple mice.

Adapted from Koehl *et al* Transplantation, 2007; 83 (5) pp. 607-614



Therefore the selection I have seen in the data presented here may have been of greater magnitude if twice daily or continuous administration of MMF is used. I have not investigated MMF/MPA levels during these experiments. When MPA levels are measured, peripheral blood serum is used and free MPA measured by high performance liquid chromatography. However, the action of MPA is intracellular and concentrations of drug within the cell may remain therapeutic despite lower levels in serum. In addition, repeated episodes of suppression may be sufficient to reduce GTP/dGTP levels to initiate apoptosis. Once programmed to undergo apoptosis, recovery of IMPDH function and GTP production will not be sufficient to reverse this programming. It has been shown that in K562 cells suppression of intracellular GTP by about 60% is required to initiate apoptosis (Meshkini et al., 2011) however the length of suppression required has not been investigated.

The effect of MMF on a cell is a result of the balance between nucleotide requirement and the activity of functional IMPDH producing guanine nucleotides. The amount of GTP/dGTP required by an individual cell will depend on its metabolic requirement especially the rate of cell division, while suppression of GTP/dGTP production by MMF will depend on the intracellular concentrations of IMPDH and drug. When I used conditions that did not provide as much of a stimulus for cell division, the requirement for GTP/dGTP would be lower and therefore higher levels of drug would be required to reduce GTP levels to a point where cell cycle arrest or apoptosis would be initiated.

This data has demonstrated a selective advantage for IMPDH2^{R} transduced cells compared to IMPDH2^{CS} transduced cells. It also demonstrates that transferred IMPDH2^{R} cells can persist during MMF treatment. Data from a

small pilot experiment not presented here, showed persistence of transferred cells in mice that were left for three weeks after 1.5Gy TBI and a two-week period of MMF therapy (100µg/g/day). IMPDH2^{R} cells remained strongly selected in mice that had received antigen and also to a lesser degree in mice that had not received antigen.

The experiments presented in this chapter were not designed to demonstrate if transferred lymphocytes retain the ability to function or whether IMPDH2^{R} transduced cells demonstrate a functional advantage for compared to control transduced cells during MMF treatment. As retention of function is a requirement for the therapeutic use of cells conferred with immunosuppressive drug resistance, I will present experiments designed to demonstrate functionality of IMPDH2^{R} transduced T cells during MMF treatment in the next chapter.

Chapter 5: Effects of MMF post-lymphodepleting irradiation and tumour control during MMF treatment

5.1 Introduction

Having demonstrated that IMPDH2^{R} transduced cells are selected *in vivo*, my subsequent experimental aim was to demonstrate the effect of MMF on the function of IMPDH2^{R} transduced cells *in vivo*. I hypothesised that, as seen *in vitro*, IMPDH2^{R} transduced cells would retain functional activity during MMF treatment following adoptive transfer.

Because treatment of PTLD during immunosuppressive therapy is one of the suggested therapeutic targets of IMPDH2^{R} transduced T cells, retention of function against tumour during MMF treatment is the focus of experiments presented in this chapter. The use of EL4 tumour cells, a lymphoma line created in C57BL6 mice exposed to 9,10-dimethyl-1,2,-benzanthracene (Gorer, 1950), is well established as an *in vivo* model of localised tumour when injected subcutaneously in C57Bl6 recipients. Variants of EL4 engineered to express a known target antigen have been developed. Having used OVA and the OT1 TCR as a model antigen system both *in vitro* and to demonstrate *in vivo* selection, I elected to use an EL4 line engineered to express OVA (EG7) (Moore et al., 1988).

In the initial experiments described by Gorer, injection of EL4 cells led to establishment of a visible tumour at day seven which then regressed spontaneously. This is due to rejection of the tumour by endogenous T or NK cells. For EG7 tumour to become established, recipient mice require lymphodepletion to prevent subsequent rejection. As described in chapter 4, when MMF and irradiation are administered together there was increased toxicity. I therefore designed protocols in which initiation of MMF therapy and

transfer of transduced therapeutic lymphocytes was delayed for several days after irradiation. My protocol was based on established protocols within our laboratory that use a single fraction of 5.5Gy total body irradiation (TBI) in C57Bl6 mice to successfully establish a subcutaneous EL4 tumour.

5.2 Aims

- To develop an *in vivo* tumour model of adoptive immunotherapy during MMF treatment.
- To investigate the effects of MMF following lymphodepleting radiation.

5.3 Results

5.3.1 Delaying initiation of MMF treatment after irradiation reduces toxicity

Initial experiments to initiate MMF five days following lymphodepleting irradiation (Single fraction, 5.5Gy) resulted in significant weight loss and death of recipients. To reduce the toxicity seen, the protocol was refined to use a reduced dose of TBI (4Gy) and to increase the window between irradiation and starting MMF to 10 days. This also ensured large established tumours at the transfer of therapeutic cells. The resulting experimental protocol is outlined (Figure 5.1). EG7 cells were suspended in matrigel and injected subcutaneously in the shaved right flank. Mice were monitored for weight loss and MMF started at a dose of 100μg/g/day on day 10. Mice were weighed daily with adjustment of the amount of MMF administered to maintain a dose of 100μg/g/day.

The percentage of baseline weight for all mice undergoing this protocol is plotted (Figure 5.2). There is no difference between MMF and vehicle treated groups. Two mice from the MMF treated groups and one mouse from the vehicle treated groups rapidly lost weight and were sacrificed on day 24 and day 17 respectively.

Figure 5.1 Overview of *in vivo* tumour model

C57Bl6/J mice were used as recipients and on day 1 were irradiated with 4Gy TBI. Four hours later 1 x 10^6 EG7 cells suspended in matrigel were injected subcutaneously in the shaved right flank of the mice. The mice were monitored for tumour growth and weight. On day 10, either MMF 100µg/g in 200µl 5% dextrose or 200µl of 5% dextrose was administered intraperitoneally. This was continued daily for 21 days with MMF dose adjusted daily for body weight. Additionally on day 10, 2.5 x 10^5 OT1 TCR Tg CD8 T cells transduced with either IMPDH2^R or IMPDH2^{CS} were injected intravenously via the tail vein. Mice were sacrificed if their weight fell below 80% of baseline or the tumour measured greater than 15mm in longest diameter.

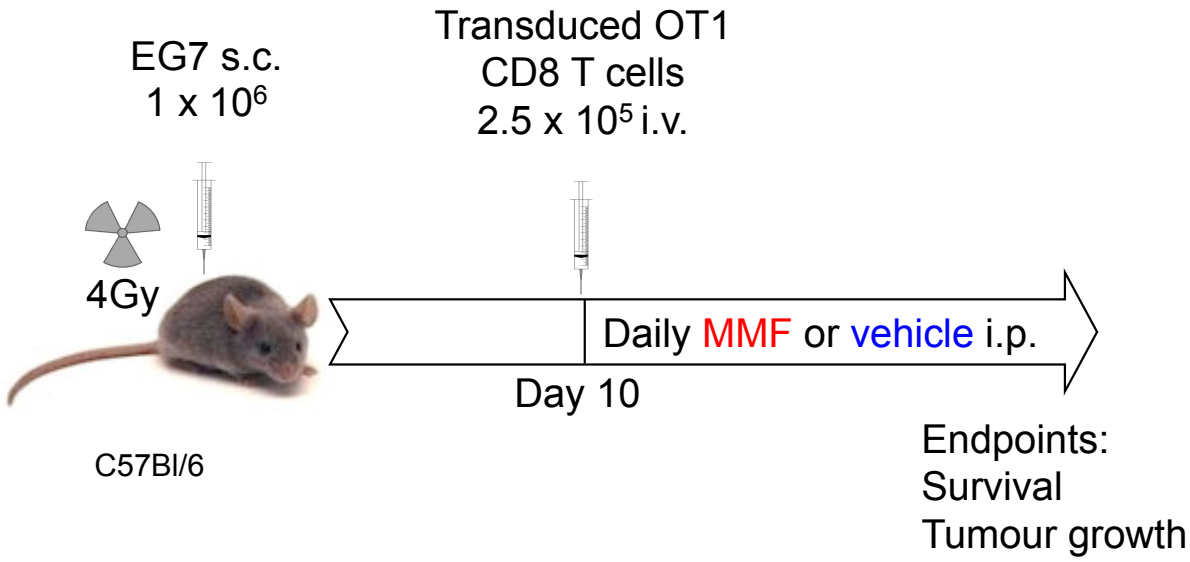
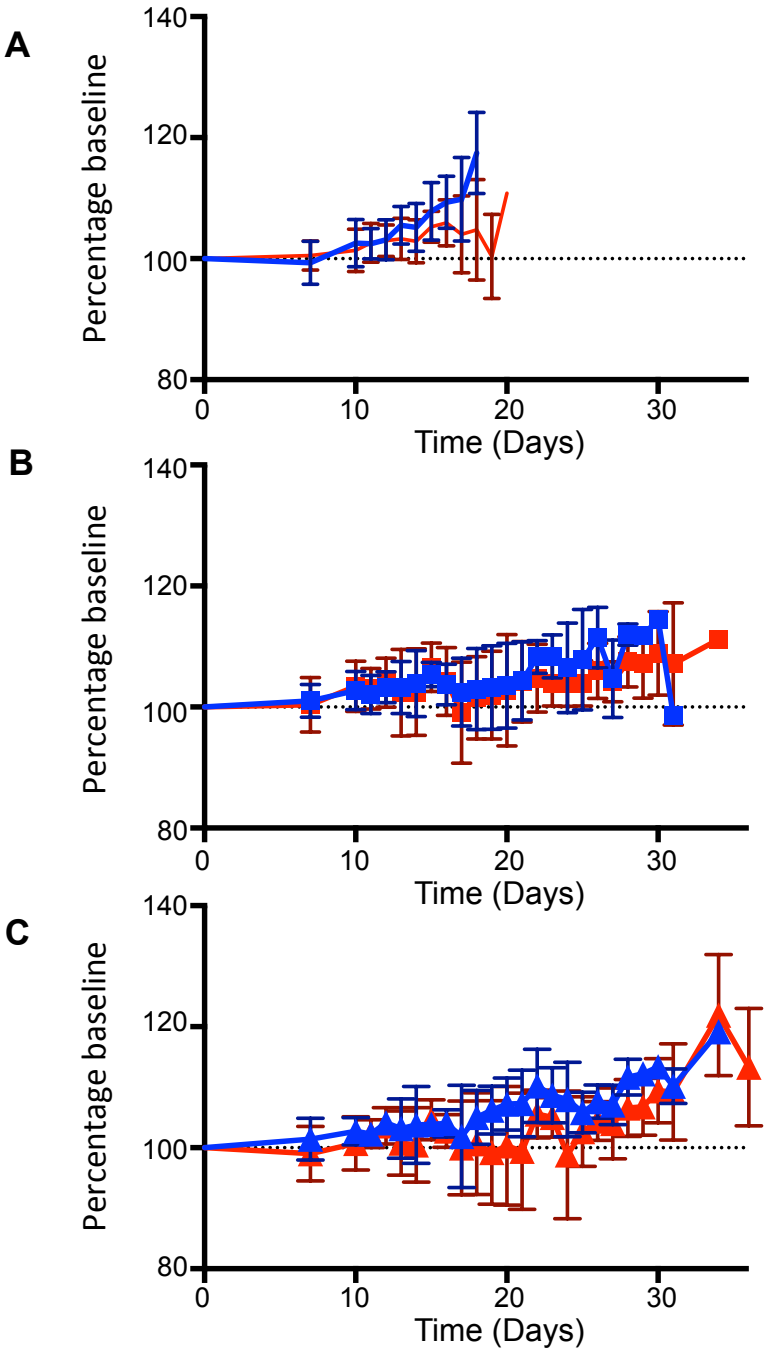


Figure 5.2 Initiating MMF treatment 10 days after lymphodepleting irradiation does not result in toxicity

Mice received 4Gy TBI, were injected subcutaneously with tumour and then rested until day 10 when daily MMF or vehicle injections were started. Mice were weighed every 1-2 days and sacrificed if weight fell below 80% of baseline. Plots showing the average weights for mice receiving no T cells (A), IMPDH2^{CS} transduced T cells (B) and IMPDH2^{R} transduced T cells (C) are shown. Results combined from 4 experiments including a total of up to 40 mice per group.



5.3.2 Combination of IMPDH2^{R} and MMF results in improved survival compared with vehicle treated mice

Mice that did not receive OT1 CD8 T cells exhibited rapid tumour growth and were sacrificed between day 16 and 20 (figures 5.3 and 5.4). The group receiving MMF alone demonstrated improved survival (log rank p=0.04) compared to mice receiving only vehicle. Both groups exhibited rapid tumour growth and median survival was one day longer with MMF compared to vehicle.

In groups receiving adoptively transferred OT1 CD8 T cells and vehicle, there was no difference between IMPDH2^{CS} or IMPDH2^{R} transduced cells. Mice in these groups required sacrifice due to tumour size between day 20 and 34 (Figure 5.3B).

Mice treated with IMPDH2^{CS} transduced OT-1 T cells and MMF exhibited significantly worse survival than those treated with vehicle (Figure 5.3D; log rank p=0.0004). There was marked variability within the vehicle treated group with rapid tumour growth in approximately 50% of recipients and the remainder surviving to similar time points as MMF treated mice. This is shown in figure 5.4B, where at day 18 there was a significant increase in tumour size in the vehicle treated group (Mann Whitney p=0.0027) but at day 26 there was no significant difference in tumour size in surviving mice (Mann-Whitney p=0.0845).

Unexpectedly, mice receiving IMPDH2^{R} transduced cells survived longer when MMF was administered compared to vehicle treated mice (Figure 5.3E; log rank p<0.0001) and showed significantly increased tumour regression (Figure 5.4C; Mann Whitney at day 18 p=0.0011; day 26 p<0.0001). I had originally hypothesised that this group would have similar tumour growth and survival as seen in vehicle treated mice.

Figure 5.3 Mice receiving IMPDH2^{R} transduced T cells and MMF exhibit increased survival over mice receiving IMPDH2^{CS} transduced cells or vehicle

Kaplain-Meyer survival curves are shown combining results from three independent experiments, total of 16 mice per groups receiving T cells and 8 mice in the no T cell groups. (A) Shows all six groups, (B) Vehicle treated groups, (C) MMF treated groups, (D) groups receiving IMPDH2^{CS} transduced cells and (E) groups receiving IMPDH2^{R} transduced cells. Log rank test MMF IMPDH2^{CS} v IMPDH2^{R} p<0.0001; IMPDH2^{R} vehicle v MMF p<0.0001; IMPDH2^{CS} Vehicle v MMF p=0.0004.

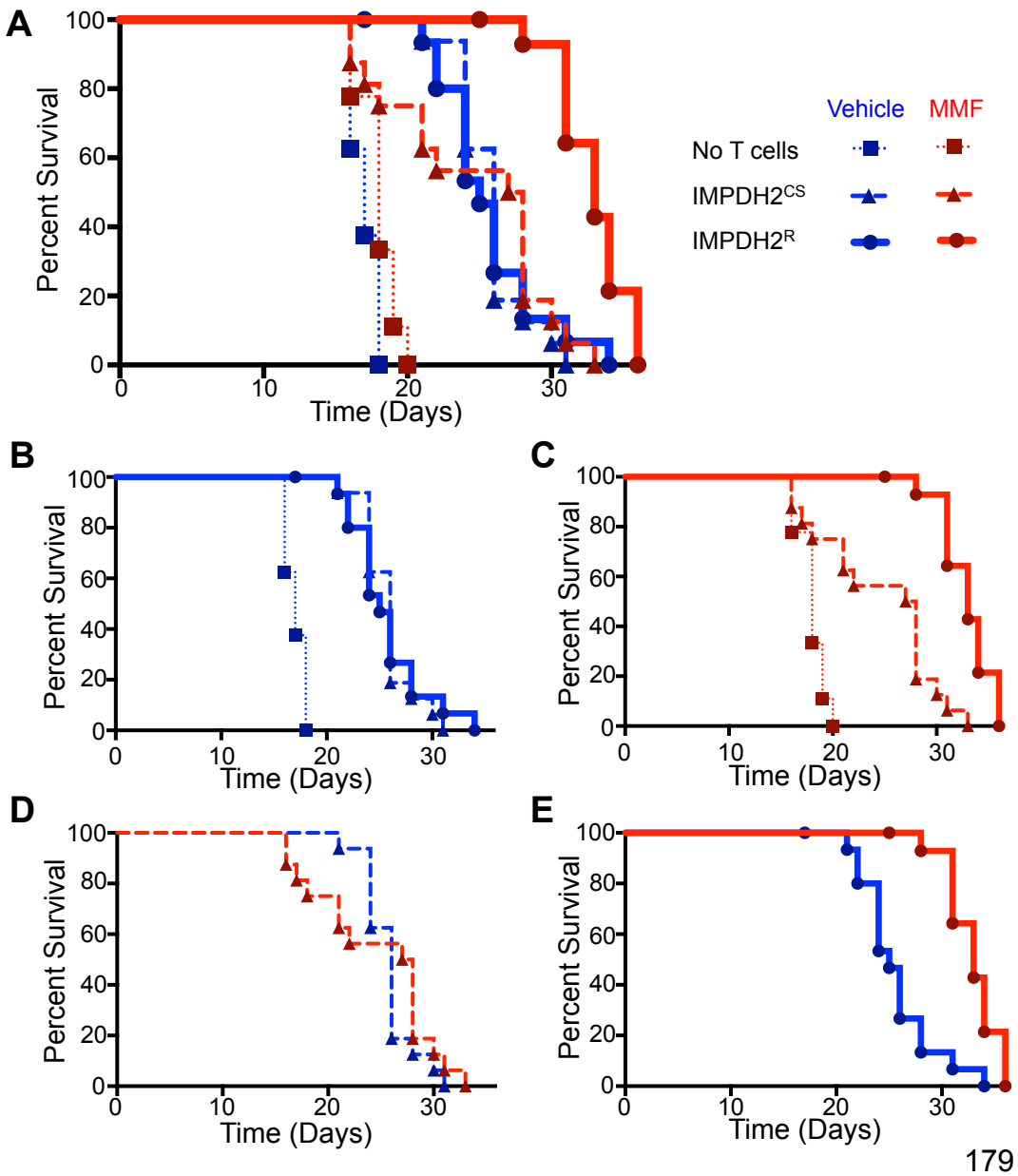
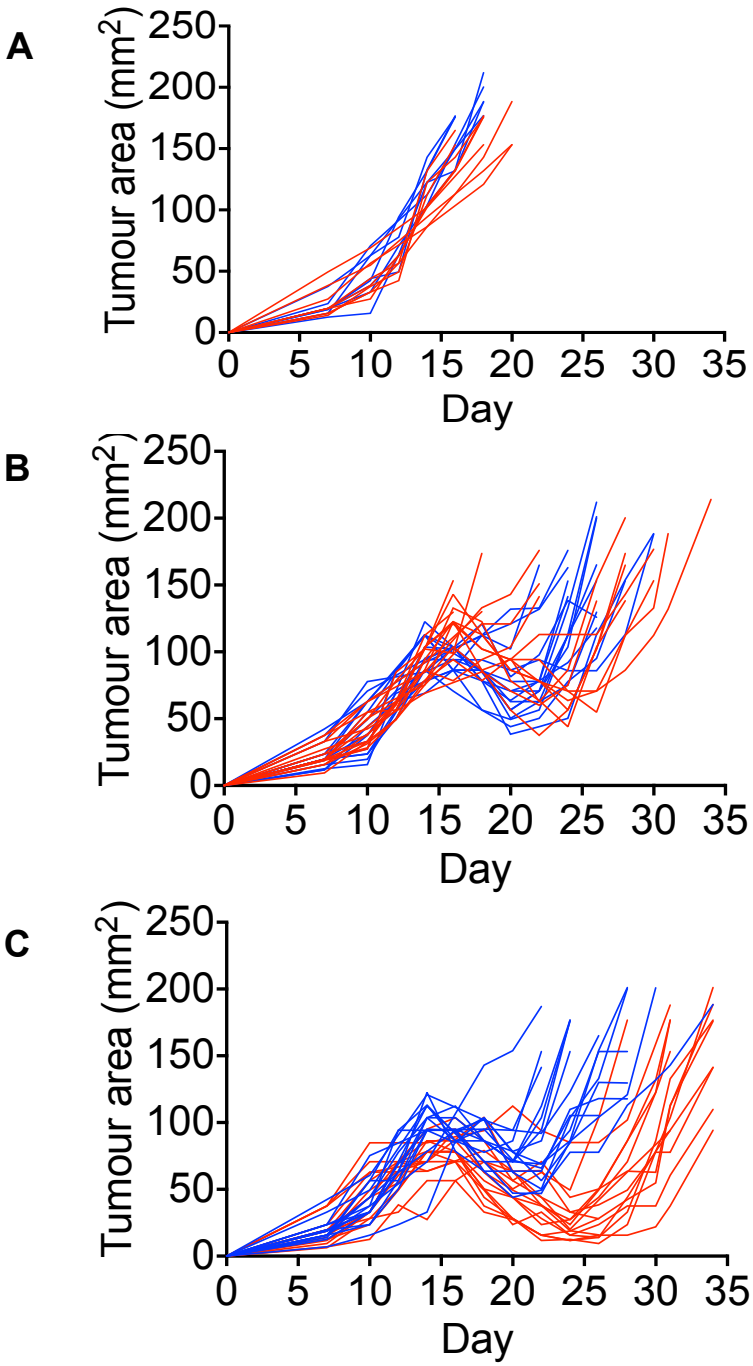


Figure 5.4 Mice receiving IMPDH2^{R} transduced cells had significantly increased tumour regression when treated with MMF compared with mice receiving IMPDH2^{CS} transduced cells or vehicle

Individual plots of tumour area for each mouse are shown for (A) groups receiving no T cells; (B) mice receiving IMPDH2^{CS} transduced cells and (C) mice receiving IMPDH2^{R} transduced cells. Mice given MMF are shown in red and vehicle in blue.



5.3.3 Mice receiving MMF and IMPDH2^{R} transduced cells have increased percentages of transduced OT1 in peripheral blood than mice receiving vehicle or IMPDH2^{CS} transduced cells

In the tumour experiments described in section 5.3.2, mice received identical numbers of OT-1 TCR CD8 T cells. The transduction efficiency in each experiment was similar for each group (IMPDH2^{CS} and IMPDH2^{R}) and ranged from 50-70% between each of the three independent experiments. Each recipient therefore received transduced (GFP positive) and untransduced (GFP negative) cells. In order to investigate the engraftment of transferred cells and their selection, surviving mice had peripheral blood sampled 10-14 days after adoptive T cell transfer (figure 5.5). Mice receiving IMPDH2^{R} cells had significantly increased percentages of both total OT1 T cells (CD8, Vα2, Vβ5 positive cells) and transduced OT1 cells than mice treated with vehicle (Mann Whitney $p<0.0001$) or IMPDH2^{CS} and MMF (Mann Whitney $p=0.0004$).

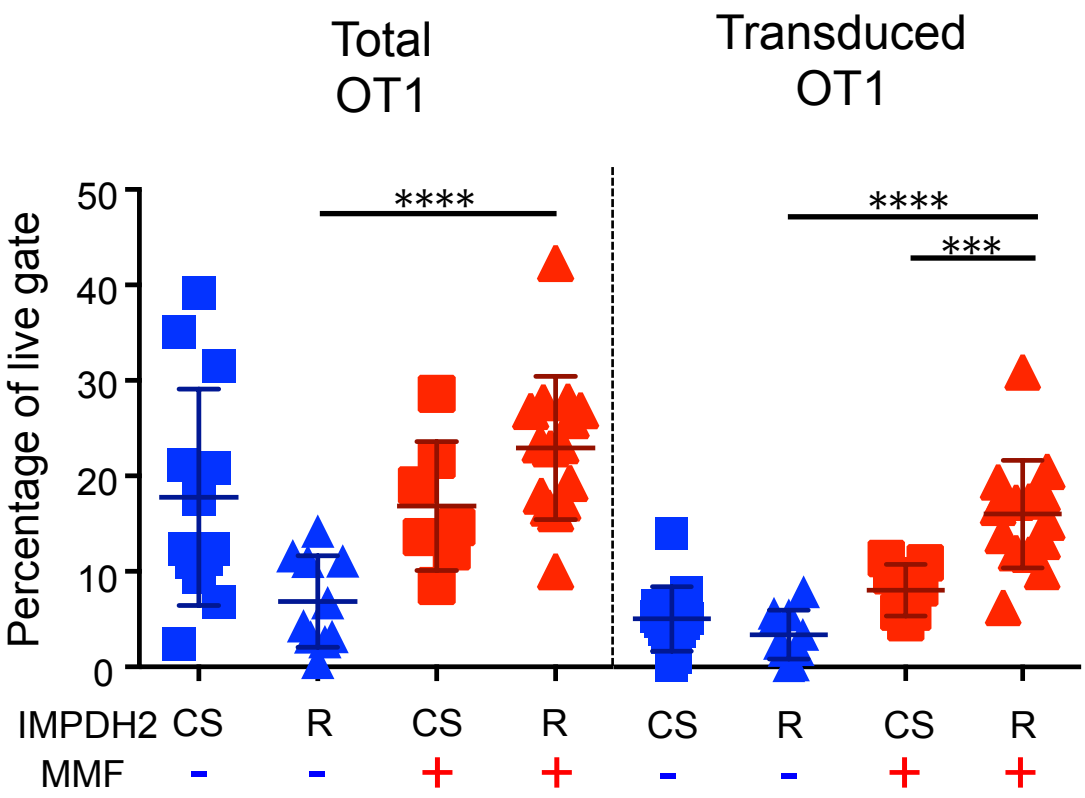
Results are given as percentages rather than absolute numbers due to the nature of sampling. However for similar volumes of blood taken from each mouse, there were notably fewer recordable events from the group receiving MMF and IMPDH2^{CS} transduced T cells in comparison to the other groups.

Following the findings presented in sections 5.3.2 and 5.3.3, I proposed the following hypothesis;

> MMF suppresses reconstitution of one or more endogenous cell type that after irradiation, in the absence of MMF, inhibits adoptively transferred cells.

Figure 5.5 Mice receiving MMF and IMPDH2^{R} transduced cells have a higher percentage of transduced OT1 T cells in peripheral blood than mice receiving vehicle or IMPDH2^{CS} transduced cells.

10-14 days after T cell transfer, peripheral blood was sampled from surviving mice. Samples were stained for CD8, TCR Vα2, TCR Vβ5 and GFP and analysed by FACS. Summary results combining three independent experiments are shown with total OT1 TCR and transduced OT1 TCR cells given as a percentage of live cells.



5.3.4 Following lymphodepleting irradiation, MMF has varying effects on reconstitution of different lymphocyte subsets in different organs

I investigated the effects of MMF on different endogenous cell types following lymphodepleting irradiation. The experimental protocol, which mirrors the tumour model described in figure 5.1 (pp. 174), is outlined in figure 5.6 and consisted of 4Gy of total body irradiation and 100μg/g/day MMF starting on day 10. Cohorts of mice were sacrificed after 5, 9 and 16 days of MMF or vehicle treatment and cells stained for B cell, T cell and NK cell markers. BrdU was added to the drinking water from day 10 to enable assessment of cell turnover. Cells were stimulated overnight with PMA and Ionomycin and intracellular staining for interferon-gamma assessed.

As had been seen in my previous experiments, the primary effect of MMF in the absence of irradiation was suppression of B cells (figure 5.7A-C). This was seen in spleen, bone marrow and lymph nodes in terms of both percentage of live cells and absolute number from day 9 of treatment (Mann-Whitney $p=0.0286$). In the spleen, there was suppression of CD4 and CD8 T cell numbers but an increase in the absolute number of NK cells at day 16. The absolute number but not relative percentage of CD4 and CD8 cells was reduced in bone marrow on day 16.

BrdU incorporation was similar for all CD4 and CD8 splenocytes tested at day 16. Surprisingly, in the MMF treated group there was greater BrdU incorporation in NK cells than in the vehicle treated group (figure 5.8A and B). The number of CD8 T cells and NK cells producing interferon gamma was significantly reduced in MMF treated groups.

Figure 5.6 Overview of model to investigate effects of MMF on different cell subsets in mice receiving lymphodepleting irradiation

B6 mice were given 4Gy of TBI and rested until day 10 when MMF 100µg/g or vehicle was administered daily via intraperitoneal injection. BrdU was added to the drinking water at a concentration of 0.8M. Mice were weighed daily and cohorts culled after 5, 9 and 16 days of MMF treatment.

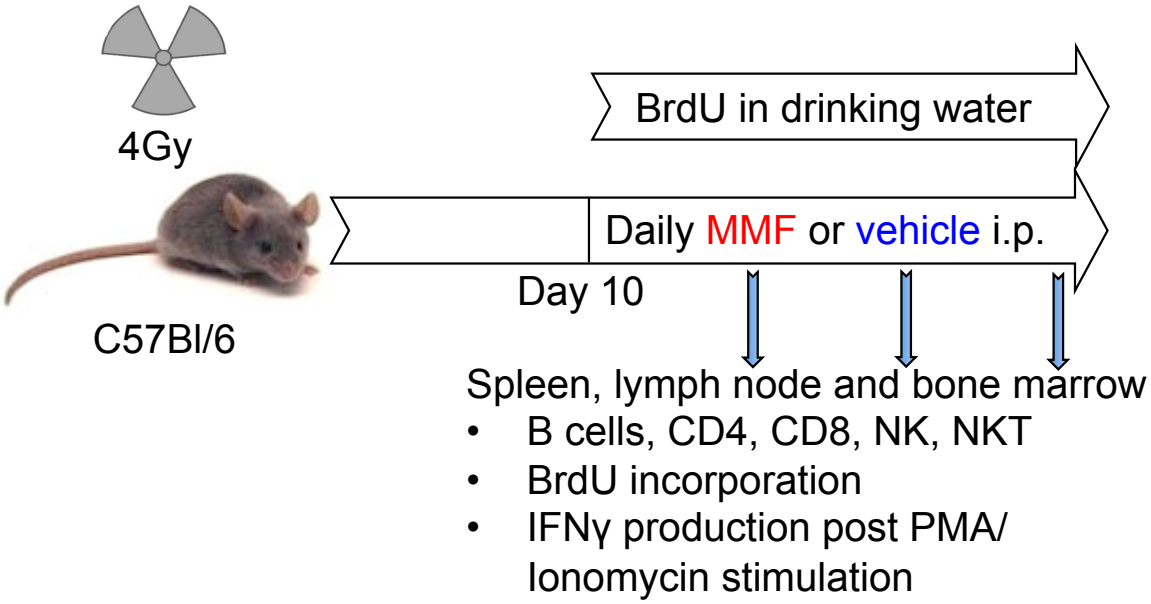


Figure 5.7 MMF has different effects on reconstitution of several lymphocyte subsets and in different organs following lymphodepleting irradiation

Mice were treated as per the model outlined in figure 5.6. Samples were stained for CD19, CD4, CD8, NK1.1 and CD335 (NKp46) and analysed by FACS. Results are given for (A) Spleen, (B) Bone marrow and (C) peripheral lymph nodes with each cell type given as a percentage of a live gate (based on forward and side scatter) and as absolute numbers. Gating was for B cells (CD19 positive), CD4 T cells (CD4 positive, NK1.1 negative), CD8 T cells (CD8 positive, NK1.1 negative), NK cells (NK1.1 and CD335 positive) and NKT cells (NK1.1 positive, CD335 negative, CD4 and/or CD8 positive). Each group consists of four mice. Blue = vehicle treated mice; red= MMF treated mice; * = Mann-Whitney test $p<0.05$.

Figure 5.7

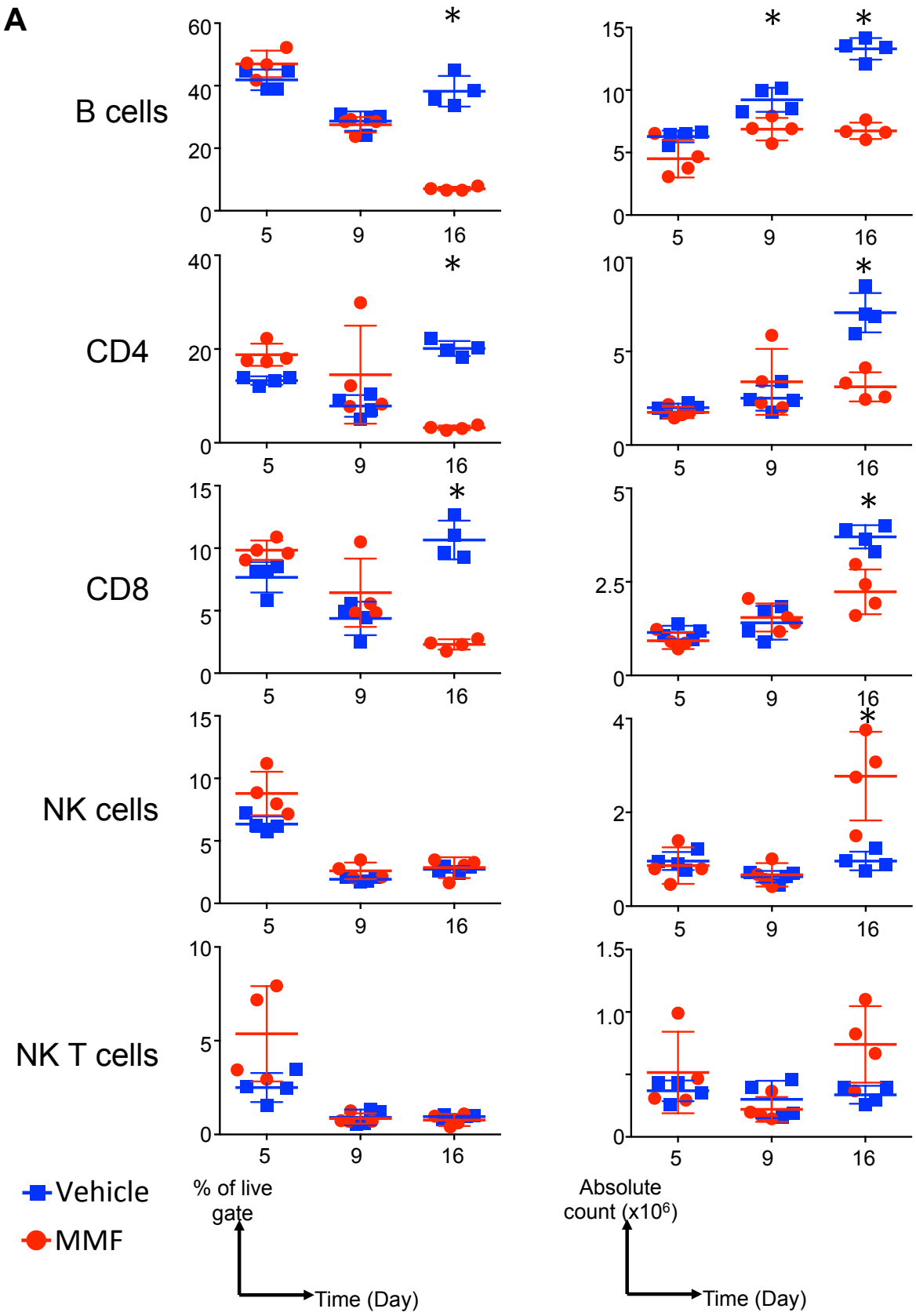

A
B cells
CD4
CD8
NK cells
NK T cells
Vehicle
MMF
% of live gate
Time (Day)
Absolute count (x10⁶)
Time (Day)

Figure 5.7

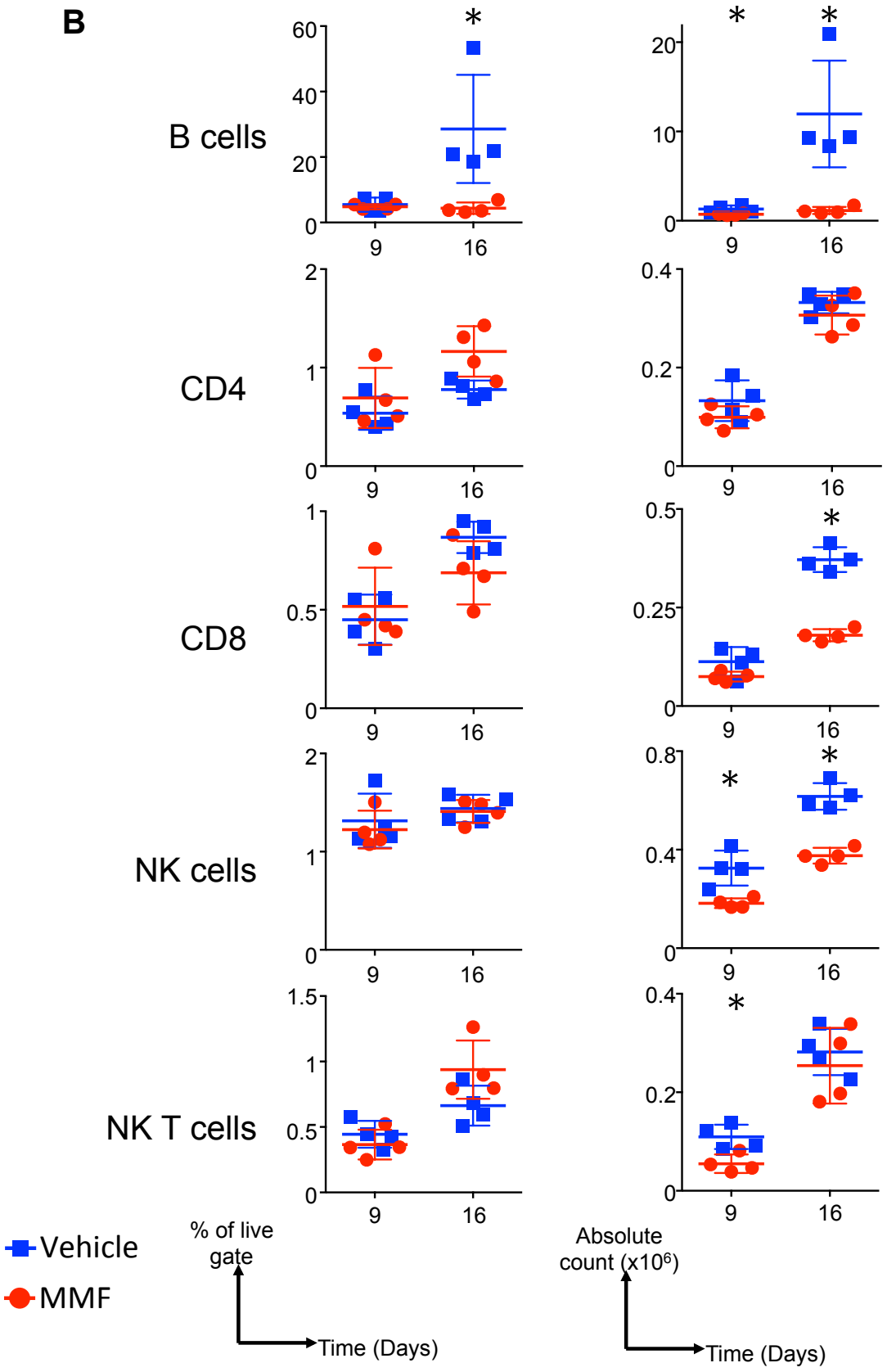

B
B cells
CD4
CD8
NK cells
NK T cells
Vehicle
MMF
% of live gate
Time (Days)
Absolute count (x10⁶)
Time (Days)

Figure 5.7

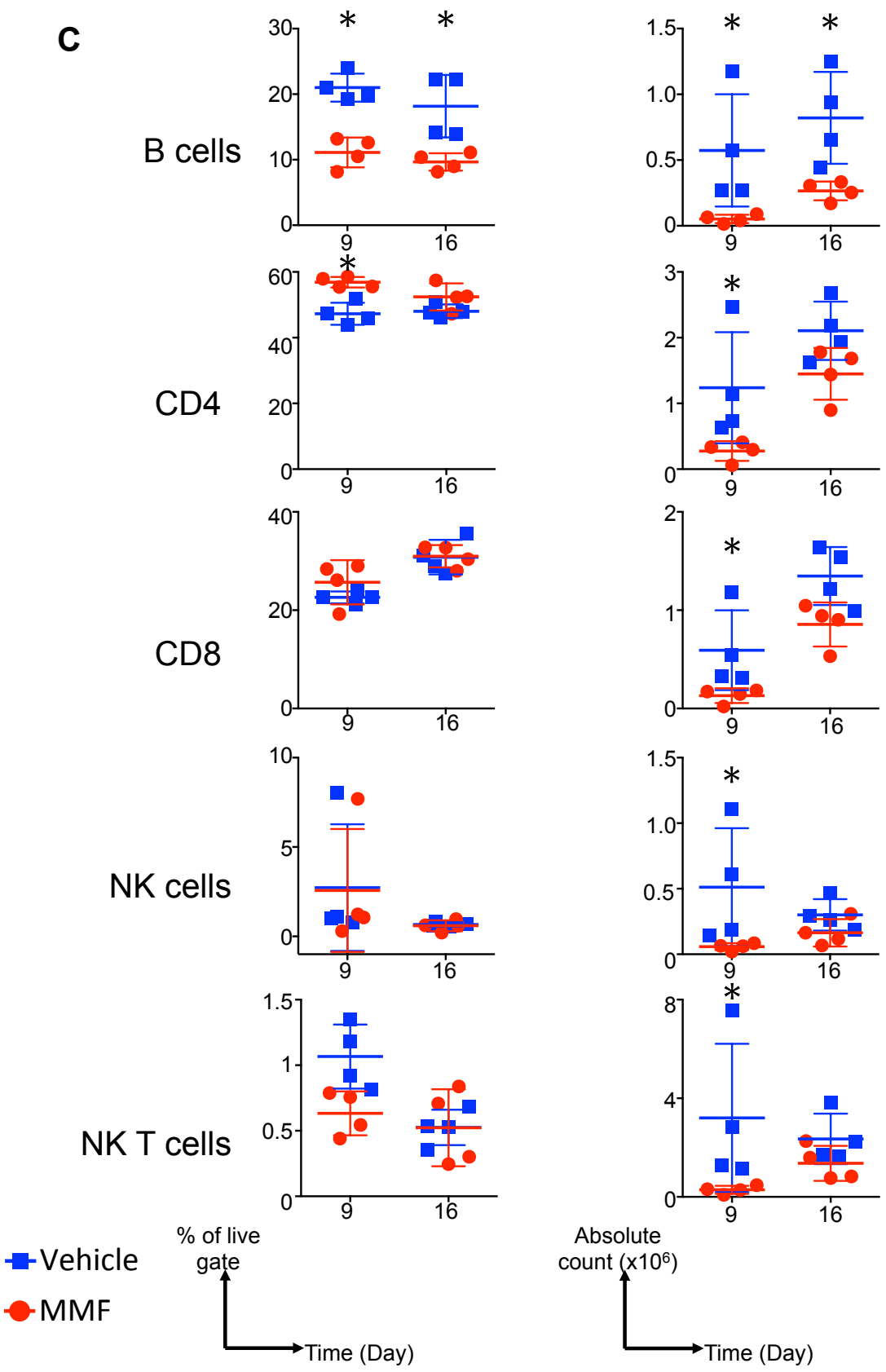
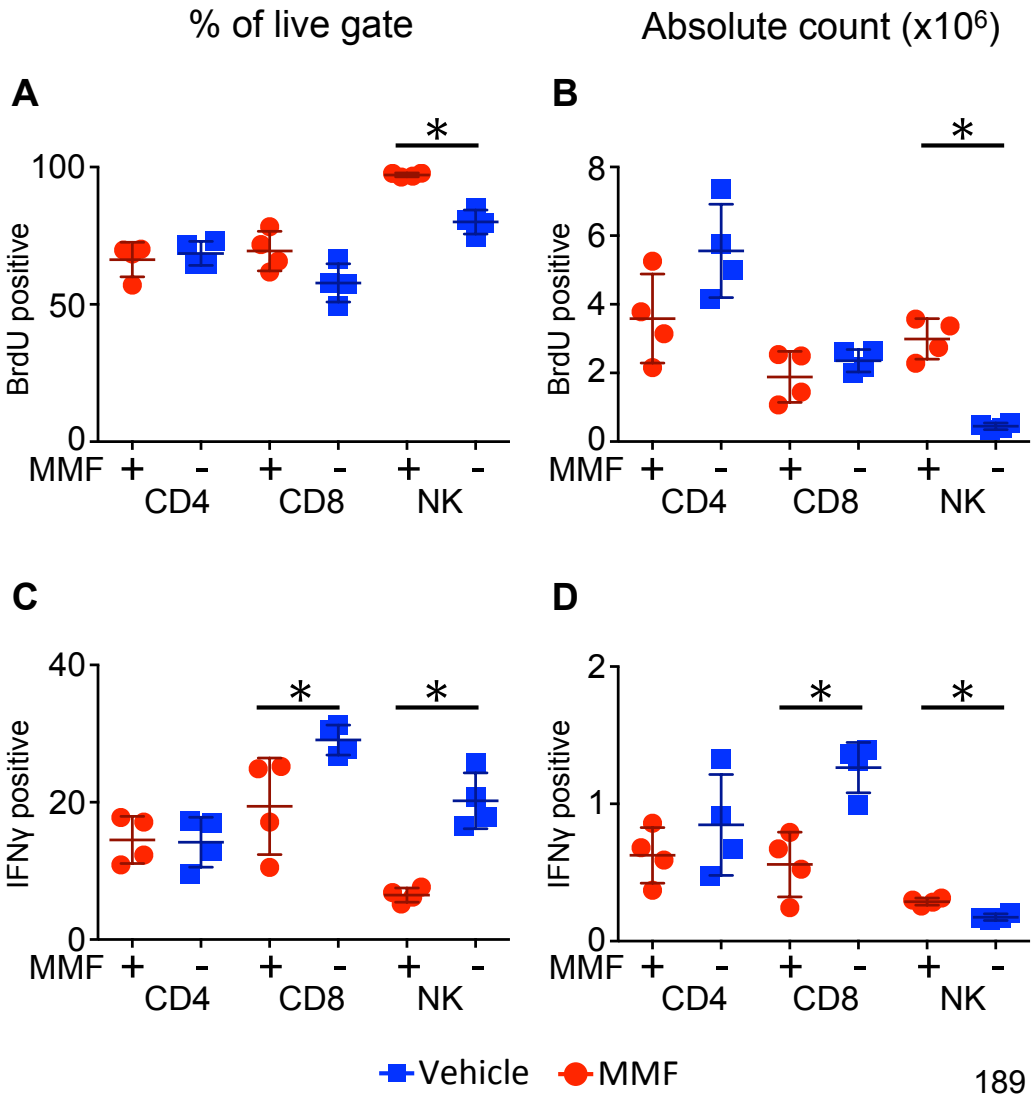

C
B cells
CD4
CD8
NK cells
NK T cells
*
30
20
10
0
60
40
3
2
1
1.5
1.0
0.5
5
8
4
9
16
% of live gate
Absolute count (x10⁶)
Time (Day)
Vehicle
MMF

Figure 5.8 MMF treatment after lymphodepleting irradiation does not significantly reduce the number of cells incorporating BrdU but does reduce the number of Interferon gamma producing CD8 and NK cells in the spleen

Splenocytes taken after 16 days of MMF treatment following 4Gy of total body irradiation were stained for CD4, CD8, NK1.1 and BrdU which had been present in drinking water since initiation of MMF. Percentage of BrdU positive cells (A) and absolute number (B) are shown. 2 x 10^6 cells from each mouse were cultured over night and stimulated with PMA and Ionomycin. The percentage (C) and absolute number (D) of Interferon gamma producing cells are shown. Each group consists of four mice. Blue = vehicle treated mice; red= MMF treated mice; * = Mann-Whitney test $p<0.05$.



5.3.5 Depletion of NK cells does not prevent improved tumour control when IMPDH2^R transduced cells and MMF are combined

NK cells are the first endogenous cells to become detectable after lymphodepletion. Previous work has shown that NK cells inhibit adoptive immunotherapy of tumour (Gattinoni, 2005). This group demonstrated that *in vivo* depletion of NK cells with PK136 antibody resulted in improved tumour control. I therefore hypothesised that improved tumour control seen when IMPDH2^R and MMF were combined resulted from a suppressive effect of MMF on endogenous NK cells. In parallel with the experiments described in section 5.3.4, I designed and performed the experiment outlined in figure 5.9. T cells were transduced with IMPDH2^R for transfer to all recipients. Four treatment groups were included; PK136 + Vehicle, PK136 + MMF, isotype control + Vehicle and isotype control + MMF.

PK136 antibody administration did not cause increased weight loss (Figure 5.10A). Immediately prior to the final dose of PK136 antibody, peripheral blood was taken to assess NK cell numbers, which were significantly reduced in the PK136 group compared to isotype control (figure 5.10B).

Individual tumour growth curves (figure 5.11) demonstrate that NK depletion did not abrogate improved tumour control with MMF. In PK136 treated mice (figure 5.11C) the MMF group had significantly smaller tumours (Mann-Whitney day 18 $p=0.0012$ and day 27 $p=0.0006$) than the vehicle treated group.

In combination with the results described in section 5.3.4, it is unlikely that suppression of endogenous reconstitution of NK cell by MMF is the mechanism by which improved tumour control is seen in mice receiving the combination of MMF and IMPDH2^R transduced T cells.

Figure 5.9 Overview of NK depletion model

C57Bl6/J mice were used as recipients and on day one were irradiated with 4Gy TBI. 4 hours later 1 x 10^6 EG7 cells suspended in matrigel were injected subcutaneously in the shaved right flank of the mice. The mice were monitored for tumour growth and weight. On day 10, either MMF 100µg/g in 200µl 5% dextrose or 200µl of 5% dextrose was administered intraperitoneally. This was continued daily for 21 days with MMF dose adjusted daily for body weight. Additionally on day 10, 2.5 x 10^5 OT1 TCR Tg CD8 T cells transduced with $IMPDH2^R$ were injected intravenously via the tail vein. Mice were sacrificed if their weight fell below 80% of baseline or the tumour measured greater than 15mm in longest diameter.

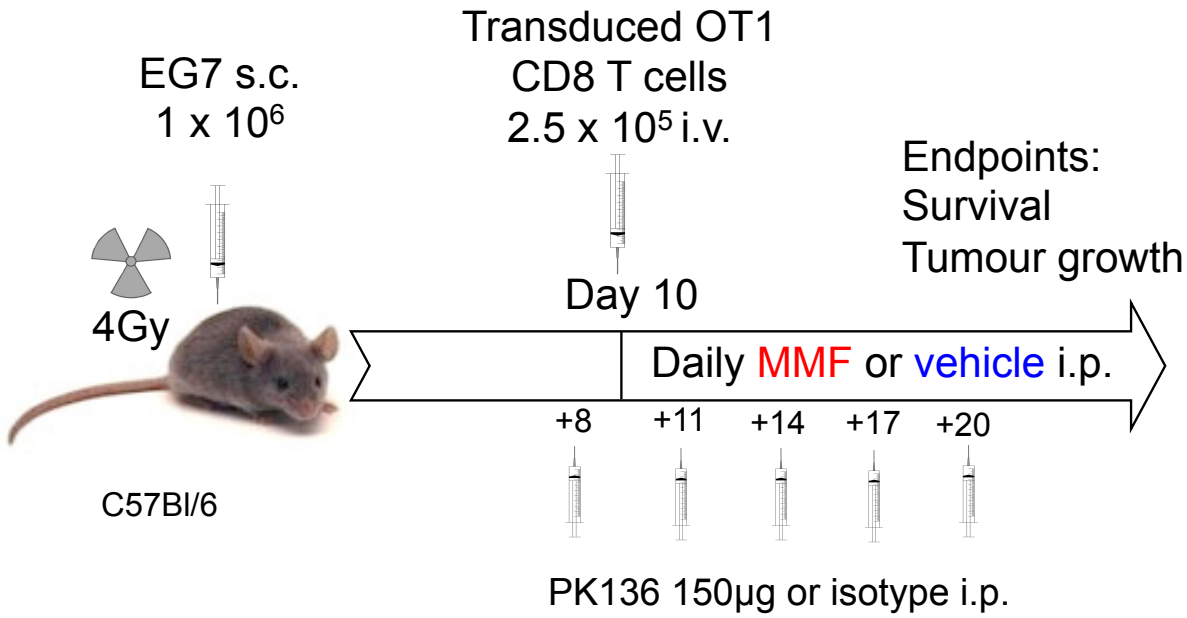


Figure 5.10 *In vivo* antibody depletion of NK cells does not cause increased toxicity

Mice treated as per the model outlined in figure 5.5 were monitored for weight loss on a two-three daily basis. Average weight change from baseline is plotted against time (A). Immediately prior to administration of the fifth and final dose of PK136, peripheral blood was taken from the mice and stained for NK1.1. The percentage of cells in a live gate (based on forward and side scatter) that were NK1.1 positive is shown (B).

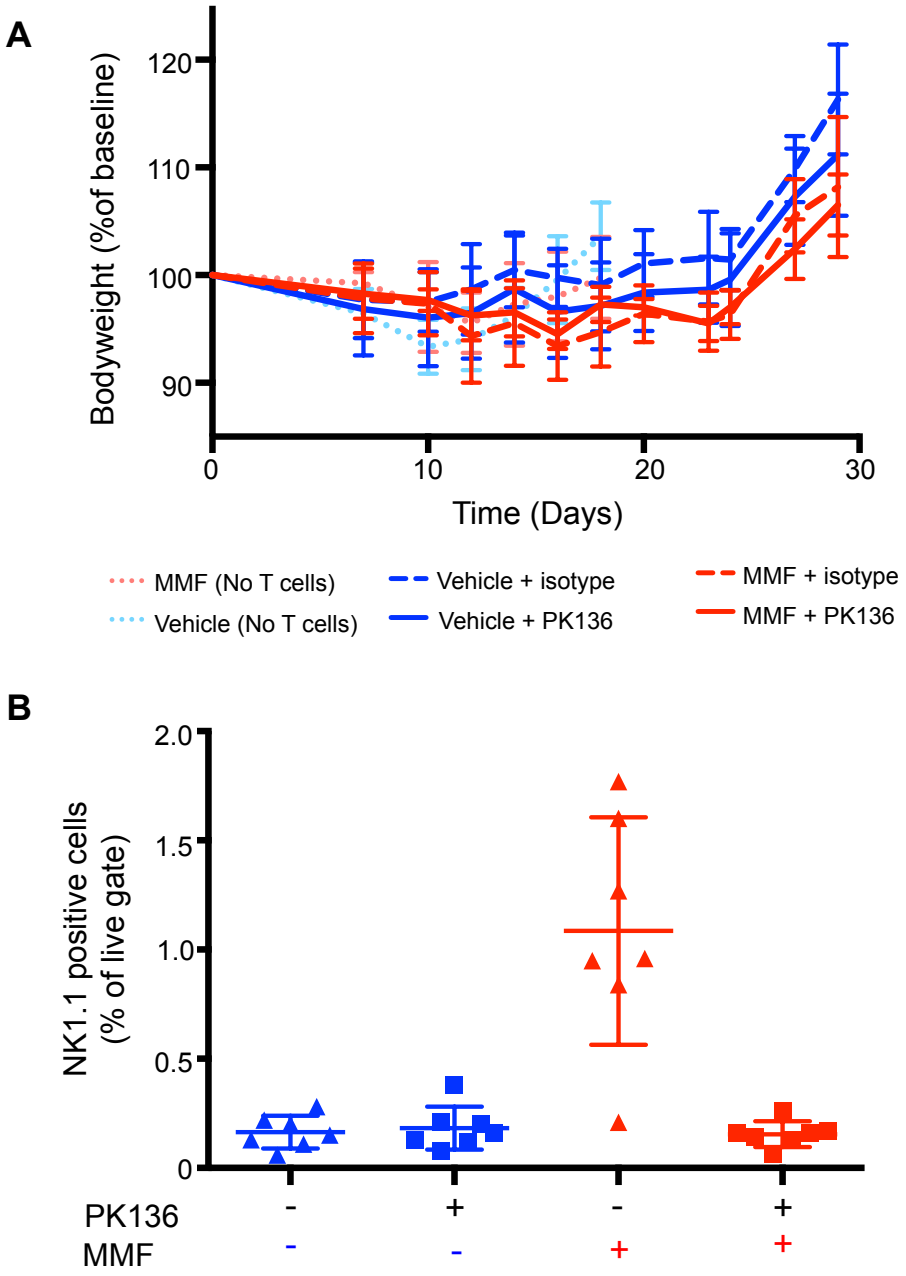
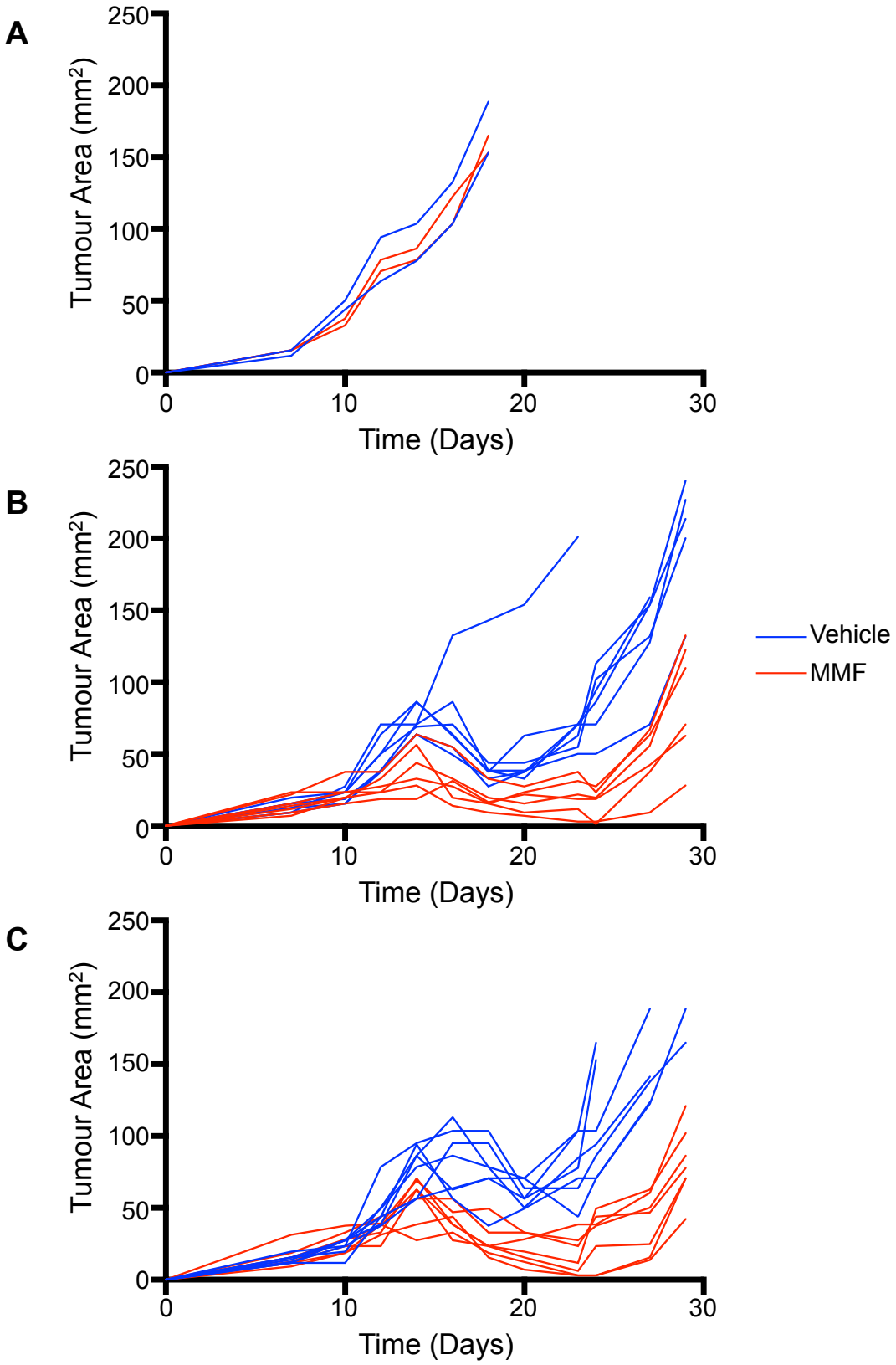


Figure 5.11 Antibody depletion of NK cells *in vivo* does not abrogate the improved tumour control seen when mice receive IMPDH2^{R} transduced cells and MMF

Mice treated as per the model outlined in figure 5.9 had tumour measured by callipers every two days during MMF or vehicle administration. Tumour area was calculated from two perpendicular diameters. Tumour growth for individual mice is plotted for (A) mice that received no T cells, (B) mice that received isotype and T cells and (C) mice that received PK136 and T cells. Each group consisted of seven mice. Mice were sacrificed when the maximum tumour diameter measured >15mm.

Figure 5.11

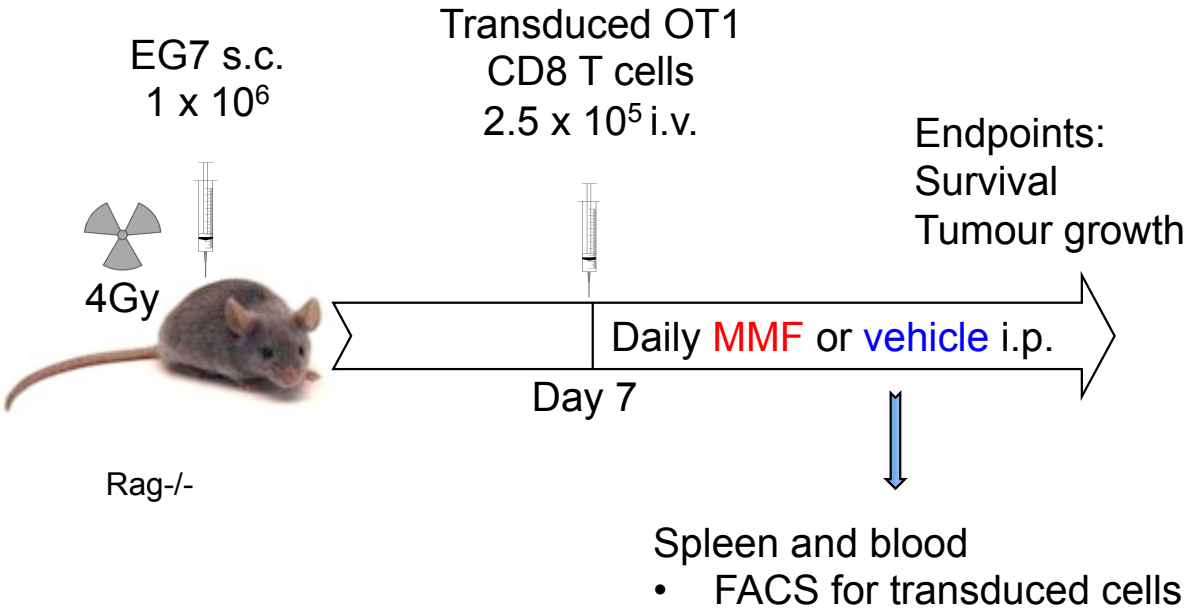

A
B
C
Tumour Area (mm²)
250
200
150
100
50
0
0
10
20
30
Time (Days)
Vehicle
MMF

5.3.6 Tumour control by IMPDH2^{R} transduced cells in the absence of endogenous lymphocytes

As suppression of endogenous cells other than NK cells could result in the improved tumour control when MMF and IMPDH2^{R} transduced cells were combined, I designed experiments using Rag knockout (Rag$^{-/-}$) mice as recipients. Rag$^{-/-}$ mice are unable to develop functional receptors required for B and T cell maturation resulting in congenital absence of lymphocytes. In an initial pilot experiment, the experiment protocol used was otherwise identical to that outlined in figure 5.1. In this experiment tumour growth was significantly faster than in previous experiments requiring sacrifice of all mice within two-three days of starting MMF. Due to the number of recipient mice available, I was unable to complete the experiments during my fellowship and the results shown are the work of Dr Pedro Velica and Sophie Ward. They followed the experimental plan I designed, with the protocol outlined in Figure 5.12. To reduce tumour growth prior to initiation of MMF, I reduced the length of time from tumour inoculation to MMF administration from 10 to 7 days. Only one mouse in the MMF alone group, who had cleared tumour, required culling due to weight loss.

Figure 5.12 Overview of Rag -/- model

Rag-/- mice were used as recipients and on day one were irradiated with 4Gy TBI. Four hours later 1 x 10^6 EG7 cells suspended in matrigel were injected subcutaneously in the shaved right flank of the mice. From day seven, either MMF 100µg/g in 200µl 5% dextrose or 200µl of 5% dextrose was administered daily intraperitoneally. This was continued with MMF dose adjusted daily for body weight. Additionally on day seven, 2.5 x 10^5 OT1 TCR Tg CD8 T cells transduced with $IMPDH2^R$ were injected intravenously via the tail vein. Mice were sacrificed if their weight fell below 80% of baseline or the tumour measured greater than 15mm in longest diameter.

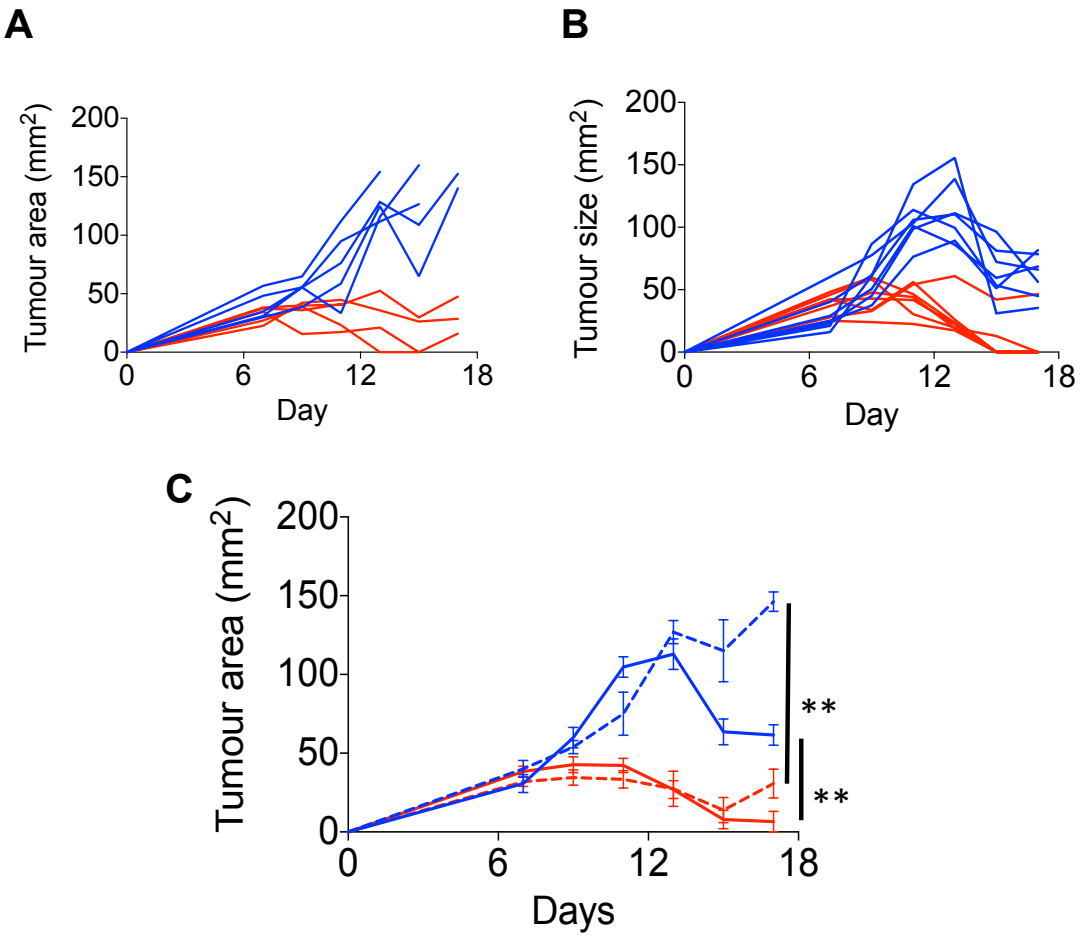


Significant tumour control was seen when MMF was given in the absence of adoptive immunotherapy compared to vehicle (Figure 5.13; Mann-Whitney p=0.0079, median 28.5mm^2 vs 152.5mm^2). In mice receiving both IMPDH2^R transduced OT-1 T cells and MMF there was no significance in tumour control as in the group receiving MMF alone (Mann-Whitney p=0.0631; median 0mm^2 vs 28.5mm^2). Of note, six of seven mice cleared tumour when both MMF and IMPDH2^R transduced cells were given while only one of five cleared tumour with MMF alone. There was significant selection of transduced cells in mice receiving MMF (data not shown).

In all mice that received vehicle, the tumour continues to grow for the first five to six days. In mice receiving vehicle and IMPDH2^R transduced OT-1 T cells, tumour growth halts from day six and then regression of tumour was observed. The tumours remained significantly larger than in the MMF treated groups (Mann Whitney p=0.0017; median 0mm^2 vs 66.03mm^2). Mice receiving no immunotherapy exhibited continued tumour growth throughout.

Figure 5.13 MMF significantly inhibits tumour growth even in the absence of adoptively transduced cells when treatment is started with less established tumours

Mice treated as per the model outlined in figure 5.12 were monitored for weight loss and tumour growth every two to three days. Individual plots of tumour area for each mouse are shown for (A) groups receiving no T cells and (B) mice receiving IMPDH2^{R} transduced cells. (C) average tumour area for each of the four groups are shown with SEM. Mice given MMF are shown in red and vehicle in blue, hashed lines indicates groups receiving no adoptive immunotherapy.



5.4 Discussion

The work presented in this chapter demonstrated that IMPDH2^{R} transduced cells retain function *in vivo* during MMF treatment. Unexpectedly, when given in combination with MMF, IMPDH2^{R} transduced cells demonstrate improved tumour control when compared to mice receiving cells and vehicle. In mice receiving the combination of MMF and IMPDH2^{R} transduced cells, I have demonstrated an increased percentage of transduced cells in the peripheral blood, due to both an increase in total transferred cell percentage and selection for IMPDH2^{R}.

There are two potential reasons for this improved outcome. Firstly that MMF suppresses an endogenous cell type enabling the transferred cells to more effectively treat the tumour. Secondly, that there is synergistic activity of both MMF and transferred cells against the tumour. Because the number of transduced cells was increased in the group receiving MMF and IMPDH2^{R} cells, I initially favoured the first hypothesis and designed experiments to investigate the role of different lymphocyte subsets.

Because NK cells are know to act as cytokine sinks and are one of the first lymphocyte subsets to reconstitute following lymphodepletion, I investigated the role of endogenous NK cells by *in vivo* antibody depletion. If NK cells were the responsible lymphocyte subset, I hypothesised that the beneficial effect of MMF would be lost. NK cell depletion did not alter the improved tumour control seen when IMPDH2^{R} transduced cells and MMF were combined.

When investigating the role of MMF on the recovery of lymphocyte subsets following lymphodepleting irradiation, I was surprised to find that the absolute

number of NK cells was increased in the spleens of mice receiving MMF compared to vehicle. The numbers of B cells and both CD4 and CD8 T cells were suppressed in the spleen at the same time point. This could be a result of differences in cellular sensitivity to MMF i.e. NK cells being less sensitive than other cells. Under conditions where cell numbers are reduced, NK cells could then out-compete for cytokines resulting in increased numbers. While NK cell numbers were increased, there was significant reduction in interferon gamma suggesting a functional deficiency. This could reflect exposure to levels of MMF able to inhibit some intracellular processes but not high enough to lead to cell cycle arrest or apoptosis.

To investigate the effect of other lymphocyte subsets, Rag -/- mice were used as recipients because they lack lymphocytes entirely. A benefit for the combination of IMPDH2^{R} transduced cells and MMF compared with vehicle persisted in the absence of lymphocytes. The results from this experiment supported the second hypothesis. In all my tumour experiments, a slight advantage had been seen for MMF in the control groups that did not receive adoptive immunotherapy. This advantage was much more pronounced in the Rag-/- experiments and produced similar results whether cells were transferred or not.

It is well established that MMF has an anti-tumour effect. This can be seen in my results using BK cells (sections 3.3.1 to 3.3.4, pp. 99-112) and is reviewed in section 1.4.6 (pp. 66). While the effect has been demonstrated easily *in vitro* against both tumour cell lines and primary malignant cells, the effect has been variable *in vivo*. Reasons for this could include the type of tumour and its location, the dosing of MMF used and differences in pharmacokinetics and

pharmacodynamics. It is possible that MMF did have a subclinical effect on tumour cells and that addition of a second therapy e.g. T cells can have a synergistic effect.

The Rag-/- experimental protocol (Figure 5.12) differed from the others described in this chapter because very rapid tumour growth was seen in a pilot experiment. Therefore at the time of initiating MMF therapy, the tumour was less well established. It is likely that in my other experiments this effect would have also been seen if a smaller tumour inoculum or the time from irradiation to adoptive therapy and MMF was reduced. The data presented here support the hypothesis that MMF and MMF-resistant adoptive immunotherapy work in synergy against experimental tumour *in vivo*.

MMF has clear effects on the reconstitution of endogenous cells following lymphodepletion. My data shows that if transferred cells are engineered with resistance to MMF, they engraft/persist at higher numbers at two weeks following transfer than both non-resistant cells given with MMF or resistant cells given alongside vehicle. The advantage gained by the transferred cells is likely due to suppression of endogenous cells, either in number or in competition for limiting cytokines.

In human studies of patients receiving HSCT, efforts to reduce the incidence of GvHD by increasing MMF dosing from 12 to 8 hourly has been shown to delay the engraftment of neutrophils from 17 to 22 days if a single unit umbilical cord is transplanted (Okamura et al., 2011). When larger stem cell doses are administered e.g. double unit umbilical cord (Ponce et al., 2014) or matched unrelated donor PBSC (Maris et al., 2004), there is no difference in time to engraftment. This likely reflects the degree of lymphoproliferation required from

the transplanted cells i.e. each cell from a smaller transferred dose will need to proliferate more and is therefore more sensitive to MMF induced suppression. Despite this effect, there was continued benefit from combining MMF and $IMPDH2^{R}$ transduced CD8 T cells both in the congenital absence of lymphocytes and depletion of NK cells. This suggests that the effect of MMF on endogenous cellular reconstitution is not responsible for the results seen.

Chapter 6: Discussion

In this work, I have demonstrated that *ex vivo* transduction of lymphocytes with IMPDH2^{R} confers MMF resistance. This resistance enables selection *in vivo* and the selected cells retain the ability to function during on going MMF exposure.

6.1 Synergy between IMPDH2^{R} transduced adoptive immunotherapy and MMF

Studies using genes conferring calcineurin-inhibitor resistance have investigated the strategy of using drug resistance genes to enable the use of adoptive immunotherapy in immunosuppressed patients post transplantation (Brewin et al., 2009, De Angelis et al., 2009). I therefore hypothesised that transduction with IMPDH2^{R} would maintain the therapeutic effect of adoptively transferred T cells during immunosuppression with MMF. My research not only supported this hypothesis but in contrast to studies utilising calcineurin-inhibitor resistance, the therapeutic effect during immunosuppression was greater when immunotherapy was combined with immunosuppression.

IMPDH2^{R} transduced cells worked in synergy with MMF to inhibit tumour. Because tumour cells utilise large amounts of guanosine nucleotides in order to proliferate it is unsurprising that many cancers express increased IMPDH (Jackson et al., 1975, Weber et al., 1981, Nagai et al., 1991, Collart et al., 1992). It has long been established that MPA has anti-tumour activity *in vitro* (Yu et al., 1989, Carter et al., 1969, Engl et al., 2005). Because MPA reduces guanosine nucleotide production, an anti-tumour effect would be expected. My initial *in vitro* findings replicated this, demonstrating that MPA increased apoptosis and cell cycle arrest resulting in reduction in the expansion of cell

numbers in several tumour cell lines including BW5147 and K562 (data not shown).

Having identified the beneficial synergistic effect of combining MMF with IMPDH2^{R} transduced cells, I hypothesised that this effect was the result of MMF induced suppression of an endogenous cell population. The expanded numbers of IMPDH2^{R} OT1 TCR Tg T cells in mice treated with MMF compared with mice given vehicle supported this hypothesis. My primary candidate for the endogenous cell type being suppressed by MMF were NK cells. Following myeloablative transplants, NK cells are the first lymphocyte subset to engraft and return to normal levels (Storek, 2008, Guo et al., 2003). They can act as cytokine sinks (Gattinoni, 2005) and directly suppress or reject transferred cells, reducing the efficacy of adoptive immunotherapy.

In experiments where NK cells were depleted *in vivo,* the additive effect of MMF and adoptive immunotherapy persisted, excluding NK cells as the responsible cell type. The involvement of other endogenous lymphocyte subsets was also excluded in experiments using Rag-/- recipients, where again the additive effect persisted. These experiments clearly demonstrated marked suppression of *in vivo* tumour growth in mice receiving MMF without adoptive immunotherapy. The original tumour experiments using C57Bl6 recipient mice had also demonstrated a statistically significant increase in length of survival in mice receiving MMF alone compared with vehicle. Tumour continued to expand during MMF treatment resulting in a 1-2 day delay before sacrifice due to tumour size compared to mice treated with vehicle alone. The protocol used in Rag-/- recipients differed from these other tumour experiments. Rapid tumour growth was seen in a pilot experiment using Rag -/- recipients, resulting in the

need to sacrifice all recipients within 2 days of adoptive immunotherapy. I subsequently reduced the time period from injection of tumour until initiation of MMF to enable the experiment to proceed.

In Rag-/- recipients, regression of tumour was immediate and was present at the first tumour measurement 2 days after starting MMF. I hypothesise that the effect was more marked in Rag -/- recipients because the subcutaneous tumours were smaller and less established at the initiation of MMF. To test this hypothesis, it would be of interest to repeat the original experiments in C57Bl6 mice with the shorter window between irradiation and initiation of adoptive immunotherapy and MMF.

Anti-tumour activity of MMF *in vitro* has not always been replicated *in vivo* (as discussed in section 1.4.6 p63). My data shows that this may potentially reflect the specific details of the model used particularly how well established the tumour is. I postulate that other tumour specific factors such as type and site of tumour at initiation of drug and MMF factors including diluent, dosage, route of administration and frequency may be the cause of the variable *in vivo* responses seen. The results I have presented here are for one tumour type in one model antigen system using a once daily intraperitoneal administration of MMF at one dose. While I have established proof of principle it would be important to confirm activity in different tumour models.

MMF is not used therapeutically as an anticancer agent. My work highlights the potential of utilising MMF in the early post transplant period both for its immunosuppressive and anti-neoplastic properties, while still enabling targeted cellular therapy.

6.2 Treatment of post-transplant viral infection with IMPDH2^{R} transduced adoptive immunotherapy

Further investigation into treatment of viral infection with IMPDH2^{R} transduced cells during MMF treatment is needed. One of the main risks of immunosuppression is viral infection. However, *in vitro* MMF has been shown to have activity against many viruses including dengue, vaccinia, herpes simplex, Coxsackie, influenza, hepatitis C, hepatitis B, human immunodeficiency virus and synergises with antiviral agents such as ganciclovir in the treatment of CMV infection. These effects are not clearly seen *in vivo*. For example there is a dose dependent increased risk of CMV disease when taking MMF, presumably because the immunosuppressive effect counteracts any direct antiviral effect. Transfer of CMV specific, IMPDH2R transduced cells could overcome the immunosuppressive effect. There is therefore a rationale to hypothesise that a synergistic effect between MMF and IMPDH2^{R} cells may be present in the treatment of viral infection as well as tumour.

6.3 Effect of MMF on endogenous lymphocytes

I had originally postulated that transduction of lymphocytes with IMPDH2^{R} would enable their use in adoptive immunotherapy in the absence of lymphodepleting irradiation or chemotherapy. I had envisaged administration of MMF alone to the recipient would create 'space' for the transferred cells enabling engraftment. While MMF primarily inhibits dividing cells via cell cycle arrest, with greater inhibition of IMPDH it disrupts cell signalling and can initiate apoptosis. Escalating the dose of MMF administered *in vivo* resulted in rapid weight loss and death of recipient mice. It became apparent in early experiments *in vivo* that, in the absence of lymphodepletion, the action of MMF

alone was insufficient to create adequate ‘space’ for engraftment either by reduction of cytokine sinks or suppression of endogenous T_{Reg} and NK cells that could suppress or kill the transferred cells.

Several of the experiments presented in this work show clear suppression of B-lymphocytes across all compartments analysed. This was seen both post lymphodepletion and when given to an otherwise untreated recipient. Suppression of B cells is reported in patients given MMF (Ganschow et al., 2001) and could be the mechanism by which MMF successfully treats antibody mediated autoimmune disease. Post lymphodepletion, the effect of MMF on the numbers of T and NK cells differed with duration of treatment and varied between the different organs studied. Interestingly NK cell numbers were increased at day 16 with increased BrdU incorporation, suggesting increased cell division. NK cell function at this time point was suppressed as evidenced by reduced production of interferon gamma in response to PMA and Ionomycin.

In vitro NK cell cultures have been shown to exhibit reduced expansion and activation (Ohata et al., 2011). In these experiments, the cultures only contained NK cells that were given sufficient levels of IL-2 and IL-15 and had constant MPA exposure. The *in vivo* environment will be significantly different with limiting cytokines and variable MPA levels. It has also been demonstrated *in vitro* that different levels of suppression of GTP result in different effects, with K562 cells undergoing differentiation with 60-70% level of GTP and induction of apoptosis when levels were suppressed below 40% of normal (Meshkini et al., 2011). I hypothesise that in inhibiting T and B cell numbers, MMF removes cytokine sinks restricting NK cell expansion. The concentration of MMF within

NK cells may be sufficient to suppress function but not to achieve apoptosis or completely block cell cycle progression.

The experiments designed to investigate lymphocyte recovery in mice receiving lymphodepleting irradiation and MMF were limited in both the number of mice and time points studied. It would be of potential interest to look more closely at different lymphocytes such as T_{Reg} and memory subsets with additional later time points to assess the final recovery during on-going MMF treatment. Another limitation was the assessment of cellular function in these experiments. I incubated cells overnight with PMA and Ionomycin in media that did not contain MPA. Cells that had been suppressed by MMF *in vivo* would have IMPDH inhibition removed for 16 hours, during which they may have sufficiently regenerated guanosine nucleotide pools to resume normal cellular function. This had the potential to overestimate function of cells from MMF treated recipients, however reduction in interferon gamma response was still demonstrated. Additionally, interferon gamma production was the only modality of function investigated and production of other effector molecules may not follow the same pattern.

6.4 Selection of IMPDH2^R transduced cells

MMF resistance, conferred by IMPDH2^R, was shown to enable selection of transduced cells both *in vitro* and *in vivo* confirming work from other groups. The combination of enhancement of the therapeutic effect and selection of transduced cells make IMPDH2^R an exciting prospect for use in all forms of adoptive immunotherapy. A plasmid designed to contain IMPDH2^R linked by a 2A sequence to either a TCR or CAR could be used to enable selection of transduced cells either prior to or after adoptive transfer. MMF resistant cells

would also express the desired TCR or CAR, therefore MMF driven selection would result in deletion of untransduced cells of unknown specificity within the product. This is particularly beneficial if the source of transduced cells is an allogeneic donor, where the untransduced population will include cells capable of causing unwanted toxicity i.e. graft versus host disease.

6.5 Safety

A concern regarding therapeutic use of genetically engineered cells is the potential for leukaemic transformation as a result of genetic manipulation. This may be due to the position that genes are inserted within genomic DNA. An additional mechanism for malignant transformation could potentially arise from over-expression of IMPDH. By increasing the level of IMPDH within a cell, increased guanine nucleotides are produced increasing proliferative potential. This is seen physiologically, following lymphocyte activation, when a large number of cells need to be produced and pathologically where increased IMPDH copy number has been identified in many tumours.

No evidence of malignant transformation was seen either *in vitro* or during *in vivo* selection experiments including where mice were left for approximately 7 weeks after transfer. I had insufficient time to perform long-term safety experiments as part of this project and therefore the risk of uncontrolled growth of transferred cells, a potential cause of significant morbidity and mortality, remains to be quantified.

Many groups have proposed the use of suicide genes or ‘kill switches’ to improve safety of transferred cells. By engineering cells with a gene that enables them to be deleted *in vivo* if they cause morbidity after transfer reduces both the consequences of uncontrolled expansion and also morbidity

from off target activation. Published examples of these suicide genes or 'kill switches' include CD20 (which enables rituximab to destroy transferred cells) (Griffioen et al., 2009, Introna et al., 2000), HSV-thymidine kinase (which enables ganciclovir to inhibit DNA synthesis) (Bonini et al., 1997) or inducible Fas or caspase (which can trigger apoptosis) (Budde et al., 2013).

6.6 Drug administration

As discussed previously (section 4.1 pp136) dosing and administration of MMF has varied between different groups. A recent publication has suggested a new strategy with *in vivo* use of MMF contained within nanoparticles that targeted the drug directly to CD4 cells using monoclonal antibodies (Look et al., 2013). This has significant advantages over the dosing strategy I used as it enables once weekly, rather than daily, administration of drug thereby significantly reducing the number of procedures recipient mice undergo. The action of MMF is also targeted more specifically at the cell type of interest and therefore higher doses of drug can be delivered to those cells without increased toxicity. Additionally, higher intracellular concentrations of MMF will promote cell death rather than cell cycle arrest. This could allow targeted removal of cytokine sinks potentially enabling engraftment in the absence of lymphodepletion.

6.7 Future experiments

As mentioned in the above discussion, there are several experiments that I have planned to carry out with cells transduced with IMPDH2^{R}. These are split into three areas, further understanding of the effect of MMF following lymphodepleting irradiation, confirmation of IMPDH2^{R} cellular function during MMF therapy and further plasmid development.

6.7.1 Effects of MMF post lymphodepletion

I would repeat the experiments described in section 5.3.4 but extend the time points to 21, 28 and 35 days. At sacrifice, I would additionally stain cells for T_{Reg} markers (CD25 and FoxP3) and T memory phenotype (CD62L CCR7, CD127 and CD44). To further investigate cellular function I would stain cells for granzyme B and perform ELISA for IL2 and IFN gamma on supernate from cells incubated with PMA and Ionomycin.

6.7.2 Confirmation of IMPDH2^R transduced cell function in other adoptive immunotherapy models

Throughout this work, I used the model antigen system OVA/OT1 TCR and demonstrated proof of principle of function of IMPDH2^R transduced cells during MMF therapy. In order to demonstrate activity against EBV+ B cell lymphomas, I propose using a protocol recently published in Blood using a calcineurin inhibitor resistance gene (Ricciardelli et al., 2014). In this model, NOD/SCID/IL2rγnull (NSG) mice inoculated subcutaneously with EBV-transformed lymphoblastoid B cell lines i.e. a human EBV+ B cell lymphoma. The tumours were labelled with F-Luc to allow imaging of tumour response to CTLs transduced with immunosuppressant drug resistance genes.

Additionally I propose studying whether IMPDH2^R transduced cells retain function against virus using a murine model of CMV infection and T cells dual transduced with a CMV TCR and IMPDH2^R. For these experiments I propose either using the MMF dosing strategy used throughout this work, namely 100µg/g/day i.p. once daily.

6.7.3 Development of new plasmids - Generation and testing of multi-immunosuppressive agent resistance plasmids

MMF is a commonly used immunosuppressive agent, particularly in solid organ transplantation. In many patients other immunosuppressive agents may be used either alone or in combination. As has already been shown by several groups, resistance to immunosuppressant will allow treatment of patients with post-transplant lymphoproliferative disorder whilst they are receiving calcineurin inhibitors. It would therefore be sensible to combine different drug resistance genes into one cassette which could then be used to produce cells resistant to all commonly used immunosuppressive drugs.

I propose generation of a vector containing IMPDH2^{R}, CNB30 (calcineurin inhibitor resistance gene) and for safety insertion of a 'kill switch'. This will need *in vitro* validation prior to investigation *in vivo* to investigate whether transduced cells will retain therapeutic efficacy in the context of dual immunosuppression. Either addition of MMF to the tumour model described in section 6.7. (Ricciardelli et al., 2014) or calcineurin inhibitor to the tumour model I developed could be used. Additional activity in models of viral infection would be beneficial. It will be important to demonstrate that the enhanced efficacy of MMF-resistant cells given with MMF persists in the context of dual immunosuppression, albeit with resistance to both immunosuppressive agents. Once safety is established I would envisage translation to use in humans for the treatment of PTLD using either selected EBV-specific or TCR transduced T cells.

References

ABRAHAM, E. P. 1945. The effect of mycophenolic acid on the growth of Staphylococcus aureus in heart broth. *Biochemical Journal,* 39**,** 398-408.

AHMADI, M., KING, J. W., XUE, S. A., VOISINE, C., HOLLER, A., WRIGHT, G. P., WAXMAN, J., MORRIS, E. & STAUSS, H. J. 2011. CD3 limits the efficacy of TCR gene therapy in vivo. *Blood,* 118**,** 3528-37.

ALEKSIC, I., BARYALEI, M., BUSCH, T., PIESKE, B., SCHORN, B., STRAUCH, J., SIRBU, H. & DALICHAU, H. 2000. Improvement of impaired renal function in heart transplant recipients treated with mycophenolate mofetil and low-dose cyclosporine. *Transplantation,* 69**,** 1586-90.

ALLEN, U., HEBERT, D., MOORE, D., DROR, Y., WASFY, S. & CANADIAN, P. S. G. 2001. Epstein-Barr virus-related post-transplant lymphoproliferative disease in solid organ transplant recipients, 1988-97: a Canadian multi-centre experience. *Pediatric Transplantation,* 5**,** 198-203.

ALLISON, A. C., ALMQUIST, S. J., MULLER, C. D. & EUGUI, E. M. 1991. In vitro immunosuppressive effects of mycophenolic acid and an ester pro-drug, RS-61443. *Transplantation Proceedings,* 23**,** 10-4.

ALLISON, A. C. & EUGUI, E. M. 1993a. The design and development of an immunosuppressive drug, mycophenolate mofetil. *Springer Seminars in Immunopathology,* 14**,** 353-380.

ALLISON, A. C. & EUGUI, E. M. 1993b. Immunosuppressive and other effects of mycophenolic acid and an ester prodrug, mycophenolate mofetil. *Immunological reviews,* 136**,** 5-28.

ALLISON, A. C. & EUGUI, E. M. 2000. Mycophenolate mofetil and its mechanisms of action. *Immunopharmacology,* 47**,** 85-118.

ANTONY, P. A., PICCIRILLO, C. A., AKPINARLI, A., FINKELSTEIN, S. E., SPEISS, P. J., SURMAN, D. R., PALMER, D. C., CHAN, C. C., KLEBANOFF, C. A., OVERWIJK, W. W., ROSENBERG, S. A. & RESTIFO, N. P. 2005. CD8+ T cell immunity against a tumor/self-antigen is augmented by CD4+ T helper cells and hindered by naturally occurring T regulatory cells. *Journal of Immunology,* 174**,** 2591-601.

APPEL, G. B., RADHAKRISHNAN, J. & GINZLER, E. M. 2005. Use of mycophenolate mofetil in autoimmune and renal diseases. *Transplantation,* 80**,** S265-71.

ASANO, M., TODA, M., SAKAGUCHI, N. & SAKAGUCHI, S. 1996. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *Journal of Experimental Medicine,* 184**,** 387-96.

AZUMA, H., BINDER, J., HEEMANN, U., SCHMID, C., TULLIUS, S. G. & TILNEY, N. L. 1995. Effects of RS61443 on functional and morphological changes in chronically rejecting rat kidney allografts. *Transplantation,* 59**,** 460-6.

BACIGALUPO, A. 2005. Antilymphocyte/thymocyte globulin for graft versus host disease prophylaxis: efficacy and side effects. *Bone Marrow Transplant,* 35**,** 225-31.

BACUS, S. S., KIGUCHI, K., CHIN, D., KING, C. R. & HUBERMAN, E. 1990. Differentiation of cultured human breast cancer cells (AU-565 and MCF-7) associated with loss of cell surface HER-2/neu antigen. *Molecular Carcinogenesis,* 3**,** 350-62.

BARYALEI, M., ZENKER, D., PIESKE, B., TONDO, K., DALICHAU, H. & ALEKSIC, I. 2003. Renal function and safety of heart transplant recipients switched to mycophenolate mofetil and low-dose cyclosporine. *Transplantation Proceedings,* 35**,** 1539-42.

BECHSTEIN, W. O., SCHILLING, M., STEELE, D. M., HULLETT, D. A. & SOLLINGER, H. W. 1993. RS-61443/cyclosporine combination therapy prolongs canine liver allograft survival. *Transplantation Proceedings,* 25**,** 702-3.

BECKER, Y. T., BECKER, B. N., PIRSCH, J. D. & SOLLINGER, H. W. 2004. Rituximab as treatment for refractory kidney transplant rejection. *Am J Transplant,* 4**,** 996-1001.

BERGER, C., TURTLE, C. J., JENSEN, M. C. & RIDDELL, S. R. 2009. Adoptive transfer of virus-specific and tumor-specific T cell immunity. *Current Opinion in Immunology,* 21**,** 224-232.

BHADURI-MCINTOSH, S., ROTENBERG, M. J., GARDNER, B., ROBERT, M. & MILLER, G. 2008. Repertoire and frequency of immune cells reactive to Epstein-Barr virus-derived autologous lymphoblastoid cell lines. *Blood,* 111**,** 1334-43.

BLUESTONE, J. A. & ABBAS, A. K. 2003. Natural versus adaptive regulatory T cells. *Nature Reviews: Immunology,* 3**,** 253-7.

BLYTH, E., CLANCY, L., SIMMS, R., GAUNDAR, S., O'CONNELL, P., MICKLETHWAITE, K. & GOTTLIEB, D. J. 2011. BK virus-specific T cells for use in cellular therapy show specificity to multiple antigens and polyfunctional cytokine responses. *Transplantation,* 92**,** 1077-84.

BLYTH, E., GAUNDAR, S. S., CLANCY, L., SIMMS, R. M., BILMON, I., MICKLETHWAITE, K. P. & GOTTLIEB, D. J. 2012. Clinical-grade varicella zoster virus-specific T cells produced for adoptive immunotherapy in hemopoietic stem cell transplant recipients. *Cytotherapy,* 14**,** 724-32.

BOEHM, I. & BIEBER, T. 2001. Chilblain lupus erythematosus Hutchinson: successful treatment with mycophenolate mofetil. *Archives of Dermatology,* 137**,** 235-6.

BOLLARD, C. M., AGUILAR, L., STRAATHOF, K. C., GAHN, B., HULS, M. H., ROUSSEAU, A., SIXBEY, J., GRESIK, M. V., CARRUM, G., HUDSON, M., DILLOO, D., GEE, A., BRENNER, M. K., ROONEY, C. M. & HESLOP, H. E. 2004. Cytotoxic T lymphocyte therapy for Epstein-Barr virus+ Hodgkin's disease. *Journal of Experimental Medicine,* 200**,** 1623-33.

BONINI, C., FERRARI, G., VERZELETTI, S., SERVIDA, P., ZAPPONE, E., RUGGIERI, L., PONZONI, M., ROSSINI, S., MAVILIO, F., TRAVERSARI, C. & BORDIGNON, C. 1997. HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia. *Science,* 276**,** 1719-24.

BOREL, J. F., FEURER, C., GUBLER, H. U. & STAHELIN, H. 1976. Biological effects of cyclosporin A: a new antilymphocytic agent. *Agents Actions,* 6**,** 468-75.

BORROWS, R., CHUSNEY, G., LOUCAIDOU, M., JAMES, A., LEE, J., TROMP, J. V., OWEN, J., CAIRNS, T., GRIFFITH, M., HAKIM, N., MCLEAN, A., PALMER, A., PAPALOIS, V. & TAUBE, D. 2006. Mycophenolic Acid 12-h Trough Level Monitoring in Renal Transplantation: Association with Acute Rejection and Toxicity. *American Journal of Transplantation,* 6**,** 121-128.

BOWNE, S. J., LIU, Q., SULLIVAN, L. S., ZHU, J., SPELLICY, C. J., RICKMAN, C. B., PIERCE, E. A. & DAIGER, S. P. 2006a. Why do mutations in the ubiquitously expressed housekeeping gene IMPDH1 cause retina-specific photoreceptor degeneration? *Investigative Ophthalmology and Visual Science,* 47**,** 3754-65.

BOWNE, S. J., SULLIVAN, L. S., MORTIMER, S. E., HEDSTROM, L., ZHU, J., SPELLICY, C. J., GIRE, A. I., HUGHBANKS-WHEATON, D., BIRCH, D. G., LEWIS, R. A., HECKENLIVELY, J. R. & DAIGER, S. P. 2006b. Spectrum and frequency of mutations in IMPDH1 associated with autosomal dominant retinitis pigmentosa and leber congenital amaurosis. *Investigative Ophthalmology and Visual Science,* 47**,** 34-42.

BRENTJENS, R., YEH, R., BERNAL, Y., RIVIERE, I. & SADELAIN, M. 2010. Treatment of chronic lymphocytic leukemia with genetically targeted autologous T cells: case report of an unforeseen adverse event in a phase I clinical trial. *Molecular Therapy,* 18**,** 666-8.

BRENTJENS, R. J., LATOUCHE, J. B., SANTOS, E., MARTI, F., GONG, M. C., LYDDANE, C., KING, P. D., LARSON, S., WEISS, M., RIVIERE, I. & SADELAIN, M. 2003. Eradication of systemic B-cell tumors by genetically targeted human T lymphocytes co-stimulated by CD80 and interleukin-15. *Nature Medicine,* 9**,** 279-86.

BRENTJENS, R. J., RIVIERE, I., PARK, J. H., DAVILA, M. L., WANG, X., STEFANSKI, J., TAYLOR, C., YEH, R., BARTIDO, S., BORQUEZ-OJEDA, O., OLSZEWSKA, M., BERNAL, Y., PEGRAM, H., PRZYBYLOWSKI, M., HOLLYMAN, D., USACHENKO, Y., PIRRAGLIA, D., HOSEY, J., SANTOS, E., HALTON, E., MASLAK, P., SCHEINBERG, D., JURCIC, J., HEANEY, M., HELLER, G., FRATTINI, M. & SADELAIN, M. 2011. Safety and persistence of adoptively transferred autologous CD19-targeted T cells in

patients with relapsed or chemotherapy refractory B-cell leukemias. *Blood,* 118**,** 4817-28.

BREWIN, J., MANCAO, C., STRAATHOF, K., KARLSSON, H., SAMARASINGHE, S., AMROLIA, P. J. & PULE, M. 2009. Generation of EBV-specific cytotoxic T cells that are resistant to calcineurin inhibitors for the treatment of posttransplantation lymphoproliferative disease. *Blood,* 114**,** 4792-4803.

BREWIN, T. B., COLE, M. P., JONES, C. T., PLATT, D. S. & TODD, I. D. 1972. Mycophenolic acid (NSC-129185): preliminary clinical trials. *Cancer Chemotherapy Reports. Part 1,* 56**,** 83-7.

BROWN, S., KONOPA, J., ZHOU, D. & THOMPSON, J. 2004. Expression of TNFalpha by CD3+ and F4/80+ cells following irradiation preconditioning and allogeneic spleen cell transplantation. *Bone Marrow Transplantation,* 33**,** 359-65.

BUDDE, L. E., BERGER, C., LIN, Y., WANG, J., LIN, X., FRAYO, S. E., BROUNS, S. A., SPENCER, D. M., TILL, B. G., JENSEN, M. C., RIDDELL, S. R. & PRESS, O. W. 2013. Combining a CD20 chimeric antigen receptor and an inducible caspase 9 suicide switch to improve the efficacy and safety of T cell adoptive immunotherapy for lymphoma. *PLoS One,* 8**,** e82742.

BULLINGHAM, R. E., NICHOLLS, A. J. & KAMM, B. R. 1998. Clinical pharmacokinetics of mycophenolate mofetil. *Clinical Pharmacokinetics,* 34**,** 429-55.

BURLINGHAM, W. J., GRAILER, A. P., HULLETT, D. A. & SOLLINGER, H. W. 1991. Inhibition of both MLC and in vitro IgG memory response to tetanus toxoid by RS-61443. *Transplantation,* 51**,** 545-7.

CANNON, M. J., SCHMID, D. S. & HYDE, T. B. 2010. Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection. *Rev Med Virol,* 20**,** 202-13.

CAPELLO, D., CERRI, M., MUTI, G., BERRA, E., ORESTE, P., DEAMBROGI, C., ROSSI, D., DOTTI, G., CONCONI, A., VIGANO, M., MAGRINI, U., IPPOLITI, G., MORRA, E., GLOGHINI, A., RAMBALDI, A., PAULLI, M., CARBONE, A. & GAIDANO, G. 2003. Molecular histogenesis of posttransplantation lymphoproliferative disorders. *Blood,* 102**,** 3775-85.

CARLTON, J. M., HIRT, R. P., SILVA, J. C., DELCHER, A. L., SCHATZ, M., ZHAO, Q., WORTMAN, J. R., BIDWELL, S. L., ALSMARK, U. C., BESTEIRO, S., SICHERITZ-PONTEN, T., NOEL, C. J., DACKS, J. B., FOSTER, P. G., SIMILLION, C., VAN DE PEER, Y., MIRANDA-SAAVEDRA, D., BARTON, G. J., WESTROP, G. D., MULLER, S., DESSI, D., FIORI, P. L., REN, Q., PAULSEN, I., ZHANG, H., BASTIDA-CORCUERA, F. D., SIMOES-BARBOSA, A., BROWN, M. T., HAYES, R. D., MUKHERJEE, M., OKUMURA, C. Y., SCHNEIDER, R., SMITH, A. J., VANACOVA, S., VILLALVAZO, M., HAAS, B. J., PERTEA, M., FELDBLYUM, T. V., UTTERBACK, T. R., SHU, C. L., OSOEGAWA, K., DE

JONG, P. J., HRDY, I., HORVATHOVA, L., ZUBACOVA, Z., DOLEZAL, P., MALIK, S. B., LOGSDON, J. M., JR., HENZE, K., GUPTA, A., WANG, C. C., DUNNE, R. L., UPCROFT, J. A., UPCROFT, P., WHITE, O., SALZBERG, S. L., TANG, P., CHIU, C. H., LEE, Y. S., EMBLEY, T. M., COOMBS, G. H., MOTTRAM, J. C., TACHEZY, J., FRASER-LIGGETT, C. M. & JOHNSON, P. J. 2007. Draft genome sequence of the sexually transmitted pathogen Trichomonas vaginalis. *Science,* 315**,** 207-12.

CARR, S. F., PAPP, E., WU, J. C. & NATSUMEDA, Y. 1993. Characterization of human type I and type II IMP dehydrogenases. *Journal of Biological Chemistry,* 268**,** 27286-27290.

CARTER, S. B., FRANKLIN, T. J., JONES, D. F., LEONARD, B. J., MILLS, S. D., TURNER, R. W. & TURNER, W. B. 1969. Mycophenolic acid: an anti-cancer compound with unusual properties. *Nature,* 223**,** 848-50.

CHANG, C. C., AVERSA, G., PUNNONEN, J., YSSEL, H. & DE VRIES, J. E. 1993. Brequinar sodium, mycophenolic acid, and cyclosporin A inhibit different stages of IL-4- or IL-13-induced human IgG4 and IgE production in vitro. *Annals of the New York Academy of Sciences,* 696**,** 108-22.

CHEADLE, E. J., GILHAM, D. E., THISTLETHWAITE, F. C., RADFORD, J. A. & HAWKINS, R. E. 2005. Killing of non-Hodgkin lymphoma cells by autologous CD19 engineered T cells. *British Journal of Haematology,* 129**,** 322-32.

CHEADLE, E. J., HAWKINS, R. E., BATHA, H., O'NEILL, A. L., DOVEDI, S. J. & GILHAM, D. E. 2010. Natural expression of the CD19 antigen impacts the long-term engraftment but not antitumor activity of CD19-specific engineered T cells. *Journal of Immunology,* 184**,** 1885-96.

CHHABRA, D., SKARO, A. I., LEVENTHAL, J. R., DALAL, P., SHAH, G., WANG, E. & GALLON, L. 2012. Long-term kidney allograft function and survival in prednisone-free regimens: tacrolimus/mycophenolate mofetil versus tacrolimus/sirolimus. *Clinical Journal of the American Society of Nephrology,* 7**,** 504-12.

CHO, B. K., RAO, V. P., GE, Q., EISEN, H. N. & CHEN, J. 2000. Homeostasis-stimulated proliferation drives naive T cells to differentiate directly into memory T cells. *Journal of Experimental Medicine,* 192**,** 549-56.

CHONG, C. R., QIAN, D. Z., PAN, F., WEI, Y., PILI, R., SULLIVAN, D. J., JR. & LIU, J. O. 2006. Identification of type 1 inosine monophosphate dehydrogenase as an antiangiogenic drug target. *Journal of Medicinal Chemistry,* 49**,** 2677-80.

CLINE, J. C., NELSON, J. D., GERZON, K., WILLIAMS, R. H. & DELONG, D. C. 1969. In vitro antiviral activity of mycophenolic acid and its reversal by guanine-type compounds. *Applied Microbiology,* 18**,** 14-20.

COCKFIELD, S. M. 2001. Identifying the patient at risk for post-transplant lymphoproliferative disorder. *Transplant Infectious Disease,* 3**,** 70-8.

COHEN, J. I. 2003. Benign and malignant Epstein-Barr virus-associated B-cell lymphoproliferative diseases. *Seminars in Hematology,* 40**,** 116-23.

COHN, R. G., MIRKOVICH, A., DUNLAP, B., BURTON, P., CHIU, S. H., EUGUI, E. & CAULFIELD, J. P. 1999. Mycophenolic acid increases apoptosis, lysosomes and lipid droplets in human lymphoid and monocytic cell lines. *Transplantation,* 68**,** 411-418.

COLBY, T. D., VANDERVEEN, K., STRICKLER, M. D., MARKHAM, G. D. & GOLDSTEIN, B. M. 1999. Crystal structure of human type II inosine monophosphate dehydrogenase: implications for ligand binding and drug design. *Proceedings of the National Academy of Sciences of the United States of America,* 96**,** 3531-3536.

COLLART, F. R., CHUBB, C. B., MIRKIN, B. L. & HUBERMAN, E. 1992. Increased inosine-5'-phosphate dehydrogenase gene expression in solid tumor tissues and tumor cell lines. *Cancer Res,* 52**,** 5826-8.

COLLART, F. R. & HUBERMAN, E. 1988. Cloning and sequence analysis of the human and Chinese hamster inosine-5'-monophosphate dehydrogenase cDNAs. *Journal of Biological Chemistry,* 263**,** 15769-15772.

COLLART, F. R. & HUBERMAN, E. 1990. Expression of IMP dehydrogenase in differentiating HL-60 cells. *Blood,* 75**,** 570-6.

COMOLI, P., GINEVRI, F., MACCARIO, R., FRASSON, C., VALENTE, U., BASSO, S., LABIRIO, M., HUANG, G. C., VERRINA, E., BALDANTI, F., PERFUMO, F. & LOCATELLI, F. 2006. Successful in vitro priming of EBV-specific CD8+ T cells endowed with strong cytotoxic function from T cells of EBV-seronegative children. *American Journal of Transplantation,* 6**,** 2169-76.

COMOLI, P., LABIRIO, M., BASSO, S., BALDANTI, F., GROSSI, P., FURIONE, M., VIGANO, M., FIOCCHI, R., ROSSI, G., GINEVRI, F., GRIDELLI, B., MORETTA, A., MONTAGNA, D., LOCATELLI, F., GERNA, G. & MACCARIO, R. 2002. Infusion of autologous Epstein-Barr virus (EBV)-specific cytotoxic T cells for prevention of EBV-related lymphoproliferative disorder in solid organ transplant recipients with evidence of active virus replication. *Blood,* 99**,** 2592-8.

COMOLI, P., MACCARIO, R., LOCATELLI, F., VALENTE, U., BASSO, S., GARAVENTA, A., TOMA, P., BOTTI, G., MELIOLI, G., BALDANTI, F., NOCERA, A., PERFUMO, F. & GINEVRI, F. 2005. Treatment of EBV-related post-renal transplant lymphoproliferative disease with a tailored regimen including EBV-specific T cells. *American Journal of Transplantation,* 5**,** 1415-22.

COOPER, L. J., TOPP, M. S., SERRANO, L. M., GONZALEZ, S., CHANG, W. C., NARANJO, A., WRIGHT, C., POPPLEWELL, L., RAUBITSCHEK, A., FORMAN, S. J. & JENSEN, M. C. 2003. T-cell clones can be rendered specific for CD19: toward the selective augmentation of the graft-versus-B-lineage leukemia effect. *Blood,* 101**,** 1637-44.

CORNUEL, J. F., MORAILLON, A. & GUERON, M. 2002. Participation of yeast inosine 5'-monophosphate dehydrogenase in an in vitro complex with a fragment of the C-rich telomeric strand. *Biochimie,* 84**,** 279-89.

COSTANZO, M. R., DIPCHAND, A., STARLING, R., ANDERSON, A., CHAN, M., DESAI, S., FEDSON, S., FISHER, P., GONZALES-STAWINSKI, G., MARTINELLI, L., MCGIFFIN, D., SMITH, J., TAYLOR, D., MEISER, B., WEBBER, S., BARAN, D., CARBONI, M., DENGLER, T., FELDMAN, D., FRIGERIO, M., KFOURY, A., KIM, D., KOBASHIGAWA, J., SHULLO, M., STEHLIK, J., TEUTEBERG, J., UBER, P., ZUCKERMANN, A., HUNT, S., BURCH, M., BHAT, G., CANTER, C., CHINNOCK, R., CRESPO-LEIRO, M., DELGADO, R., DOBBELS, F., GRADY, K., KAO, W., LAMOUR, J., PARRY, G., PATEL, J., PINI, D., TOWBIN, J., WOLFEL, G., DELGADO, D., EISEN, H., GOLDBERG, L., HOSENPUD, J., JOHNSON, M., KEOGH, A., LEWIS, C., O'CONNELL, J., ROGERS, J., ROSS, H., RUSSELL, S., VANHAECKE, J., INTERNATIONAL SOCIETY OF, H. & LUNG TRANSPLANTATION, G. 2010. The International Society of Heart and Lung Transplantation Guidelines for the care of heart transplant recipients. *J Heart Lung Transplant,* 29**,** 914-56.

CRABTREE, G. W. & HENDERSON, J. F. 1971. Rate-limiting steps in the interconversion of purine ribonucleotides in Ehrlich ascites tumor cells in vitro. *Cancer Research,* 31**,** 985-91.

DAVIS, S. & RAMBOTTI, P. 1982. Adenosine deaminase, nucleoside phosphorylase and hypoxanthine-guanine phosphoribosyltransferase activity in normal lymphocyte subpopulations. *Anticancer Res,* 2**,** 125-8.

DE ANGELIS, B., DOTTI, G., QUINTARELLI, C., HUYE, L. E., ZHANG, L., ZHANG, M., PANE, F., HESLOP, H. E., BRENNER, M. K. & ROONEY, C. M. 2009. Generation of Epstein-Barr virus–specific cytotoxic T lymphocytes resistant to the immunosuppressive drug tacrolimus (FK506). *Blood,* 114**,** 4784-4791.

DE BRUIJN, M. L. H., SCHUMACHER, T. N. M., NIELAND, J. D., PLOEGH, H. L., MARTIN KAST, W. & MELIEF, C. J. M. 1991. Peptide loading of empty major histocompatibility complex molecules on RMA-S cells allows the induction of primary cytotoxic T lymphocyte responses. *European Journal of Immunology,* 21**,** 2963-2970.

DEIERHOI, M. H., SOLLINGER, H. W., DIETHELM, A. G., BELZER, F. O. & KAUFFMAN, R. S. 1993. One-year follow-up results of a phase I trial of mycophenolate mofetil (RS61443) in cadaveric renal transplantation. *Transplantation Proceedings,* 25**,** 693-4.

DEMBIC, Z., HAAS, W., WEISS, S., MCCUBREY, J., KIEFER, H., VON BOEHMER, H. & STEINMETZ, M. 1986. Transfer of specificity by murine alpha and beta T-cell receptor genes. *Nature,* 320**,** 232-8.

DICTIONARY, A. H. 2004. The American Heritage Medical Dictionary. *Houghton Mifflin Company*.

DOMHAN, S., MUSCHAL, S., SCHWAGER, C., MORATH, C., WIRKNER, U., ANSORGE, W., MAERCKER, C., ZEIER, M., HUBER, P. E. & ABDOLLAHI, A. 2008. Molecular mechanisms of the antiangiogenic and antitumor effects of mycophenolic acid. *Molecular Cancer Therapeutics,* 7**,** 1656-1668.

DOMINGO-DOMENECH, E., DE SANJOSE, S., GONZALEZ-BARCA, E., ROMAGOSA, V., DOMINGO-CLAROS, A., GIL-VERNET, S., FIGUERAS, J., MANITO, N., OTON, B., PETIT, J., GRANENA, A. & FERNANDEZ DE SEVILLA, A. 2001. Post-transplant lymphomas: a 20-year epidemiologic, clinical and pathologic study in a single center. *Haematologica,* 86**,** 715-21.

DOUBROVINA, E., OFLAZ-SOZMEN, B., PROCKOP, S. E., KERNAN, N. A., ABRAMSON, S., TERUYA-FELDSTEIN, J., HEDVAT, C., CHOU, J. F., HELLER, G., BARKER, J. N., BOULAD, F., CASTRO-MALASPINA, H., GEORGE, D., JAKUBOWSKI, A., KOEHNE, G., PAPADOPOULOS, E. B., SCARADAVOU, A., SMALL, T. N., KHALAF, R., YOUNG, J. W. & O'REILLY, R. J. 2012. Adoptive immunotherapy with unselected or EBV-specific T cells for biopsy-proven EBV+ lymphomas after allogeneic hematopoietic cell transplantation. *Blood,* 119**,** 2644-56.

DUDLEY, M. E. 2002. Cancer Regression and Autoimmunity in Patients After Clonal Repopulation with Antitumor Lymphocytes. *Science,* 298**,** 850-854.

DUDLEY, M. E. 2005. Adoptive Cell Transfer Therapy Following Non-Myeloablative but Lymphodepleting Chemotherapy for the Treatment of Patients With Refractory Metastatic Melanoma. *Journal of Clinical Oncology,* 23**,** 2346-2357.

DUDLEY, M. E., YANG, J. C., SHERRY, R., HUGHES, M. S., ROYAL, R., KAMMULA, U., ROBBINS, P. F., HUANG, J., CITRIN, D. E., LEITMAN, S. F., WUNDERLICH, J., RESTIFO, N. P., THOMASIAN, A., DOWNEY, S. G., SMITH, F. O., KLAPPER, J., MORTON, K., LAURENCOT, C., WHITE, D. E. & ROSENBERG, S. A. 2008. Adoptive Cell Therapy for Patients With Metastatic Melanoma: Evaluation of Intensive Myeloablative Chemoradiation Preparative Regimens. *Journal of Clinical Oncology,* 26**,** 5233-5239.

DUMMER, W., ERNST, B., LEROY, E., LEE, D. & SURH, C. 2001. Autologous regulation of naive T cell homeostasis within the T cell compartment. *Journal of Immunology,* 166**,** 2460-8.

DUMMER, W., NIETHAMMER, A. G., BACCALA, R., LAWSON, B. R., WAGNER, N., REISFELD, R. A. & THEOFILOPOULOS, A. N. 2002. T cell homeostatic proliferation elicits effective antitumor autoimmunity. *Journal of Clinical Investigation,* 110**,** 185-92.

EBERHARD, O. K., KLIEM, V. & BRUNKHORST, R. 1999. Five cases of Kaposi's sarcoma in kidney graft recipients: possible influence of the immunosuppressive therapy. *Transplantation,* 67**,** 180-4.

EICKENBERG, S., MICKHOLZ, E., JUNG, E., NOFER, J. R., PAVENSTADT, H. J. & JACOBI, A. M. 2012. Mycophenolic acid counteracts B cell

proliferation and plasmablast formation in patients with systemic lupus erythematosus. *Arthritis Res Ther,* 14**,** R110.

EINSELE, H., ROOSNEK, E., RUFER, N., SINZGER, C., RIEGLER, S., LÖFFLER, J., GRIGOLEIT, U., MORIS, A., RAMMENSEE, H. G. & KANZ, L. 2002. Infusion of cytomegalovirus (CMV)–specific T cells for the treatment of CMV infection not responding to antiviral chemotherapy. *Blood,* 99**,** 3916-3922.

EKBERG, H., TEDESCO-SILVA, H., DEMIRBAS, A., VITKO, S., NASHAN, B., GURKAN, A., MARGREITER, R., HUGO, C., GRINYO, J. M., FREI, U., VANRENTERGHEM, Y., DALOZE, P., HALLORAN, P. F. & STUDY, E. L.-S. 2007. Reduced exposure to calcineurin inhibitors in renal transplantation. *New England Journal of Medicine,* 357**,** 2562-75.

ENGL, T., MAKAREVIC, J., RELJA, B., NATSHEH, I., MULLER, I., BEECKEN, W. D., JONAS, D. & BLAHETA, R. A. 2005. Mycophenolate mofetil modulates adhesion receptors of the beta1 integrin family on tumor cells: impact on tumor recurrence and malignancy. *BMC Cancer,* 5**,** 4.

ENGSTRAND, M., LIDEHALL, A. K., TOTTERMAN, T. H., HERRMAN, B., ERIKSSON, B. M. & KORSGREN, O. 2003. Cellular responses to cytomegalovirus in immunosuppressed patients: circulating CD8+ T cells recognizing CMVpp65 are present but display functional impairment. *Clinical and Experimental Immunology,* 132**,** 96-104.

ERNST, B., LEE, D. S., CHANG, J. M., SPRENT, J. & SURH, C. D. 1999. The peptide ligands mediating positive selection in the thymus control T cell survival and homeostatic proliferation in the periphery. *Immunity,* 11**,** 173-81.

ESQUIVEL, F., YEWDELL, J. & BENNINK, J. 1992. RMA/S cells present endogenously synthesized cytosolic proteins to class I-restricted cytotoxic T lymphocytes. *The Journal of experimental medicine,* 175**,** 163-168.

EUGUI, E. M., ALMQUIST, S. J., MULLER, C. D. & ALLISON, A. C. 1991a. Lymphocyte-selective cytostatic and immunosuppressive effects of mycophenolic acid in vitro: role of deoxyguanosine nucleotide depletion. *Scandinavian journal of immunology,* 33**,** 161-173.

EUGUI, E. M., MIRKOVICH, A. & ALLISON, A. C. 1991b. Lymphocyte-selective antiproliferative and immunosuppressive effects of mycophenolic acid in mice. *Scandinavian journal of immunology,* 33**,** 175-183.

EUROPEAN MYCOPHENOLATE MOFETIL COOPERATIVE STUDY GROUP 1995. Placebo-controlled study of mycophenolate mofetil combined with cyclosporin and corticosteroids for prevention of acute rejection. . *Lancet,* 345**,** 1321-5.

FARAZI, T., LEICHMAN, J., HARRIS, T., CAHOON, M. & HEDSTROM, L. 1997. Isolation and characterization of mycophenolic acid-resistant mutants of inosine-5'-monophosphate dehydrogenase. *Journal of Biological Chemistry,* 272**,** 961-965.

FERRARA, J. L., LEVINE, J. E., REDDY, P. & HOLLER, E. 2009. Graft-versus-host disease. *Lancet,* 373**,** 1550-61.

FISSON, S., DARRASSE-JEZE, G., LITVINOVA, E., SEPTIER, F., KLATZMANN, D., LIBLAU, R. & SALOMON, B. L. 2003. Continuous activation of autoreactive CD4+ CD25+ regulatory T cells in the steady state. *Journal of Experimental Medicine,* 198**,** 737-46.

FLEMING, M. A., CHAMBERS, S. P., CONNELLY, P. R., NIMMESGERN, E., FOX, T., BRUZZESE, F. J., HOE, S. T., FULGHUM, J. R., LIVINGSTON, D. J., STUVER, C. M., SINTCHAK, M. D., WILSON, K. P. & THOMSON, J. A. 1996. Inhibition of IMPDH by mycophenolic acid: dissection of forward and reverse pathways using capillary electrophoresis. *Biochemistry,* 35**,** 6990-6997.

FLOREY, H. W., JENNINGS, M. A. & ET AL. 1946. Mycophenolic acid; an antibiotic from Penicillium brevicompactum Dlerckx. *Lancet,* 247**,** 46-9.

FLORYK, D. & HUBERMAN, E. 2006. Mycophenolic acid-induced replication arrest, differentiation markers and cell death of androgen-independent prostate cancer cells DU145. *Cancer Letters,* 231**,** 20-9.

FLORYK, D., TOLLAKSEN, S. L., GIOMETTI, C. S. & HUBERMAN, E. 2004. Differentiation of human prostate cancer PC-3 cells induced by inhibitors of inosine 5'-monophosphate dehydrogenase. *Cancer Research,* 64**,** 9049-56.

FRANKLIN, T. J. & COOK, J. M. 1969. The inhibition of nucleic acid synthesis by mycophenolic acid. *Biochemical Journal,* 113**,** 515-524.

FRANKLIN, T. J., JACOBS, V., JONES, G., PLE, P. & BRUNEAU, P. 1996. Glucuronidation associated with intrinsic resistance to mycophenolic acid in human colorectal carcinoma cells. *Cancer Research,* 56**,** 984-7.

FUJINO, Y., KAWAMURA, T., HULLETT, D. A. & SOLLINGER, H. W. 1994. Evaluation of cyclosporine, mycophenolate mofetil, and Brequinar sodium combination therapy on hamster-to-rat cardiac xenotransplantation. *Transplantation,* 57**,** 41-6.

FUTER, O., SINTCHAK, M. D., CARON, P. R., NIMMESGERN, E., DECENZO, M. T., LIVINGSTON, D. J. & RAYBUCK, S. A. 2002. A mutational analysis of the active site of human type II inosine 5'-monophosphate dehydrogenase. *Biochimica et biophysica acta,* 1594**,** 27-39.

GAN, L., PETSKO, G. A. & HEDSTROM, L. 2002. Crystal structure of a ternary complex of Tritrichomonas foetus inosine 5'-monophosphate dehydrogenase: NAD+ orients the active site loop for catalysis. *Biochemistry,* 41**,** 13309-17.

GANSCHOW, R., LYONS, M., KEMPER, M. J. & BURDELSKI, M. 2001. B-cell dysfunction and depletion using mycophenolate mofetil in a pediatric combined liver and kidney graft recipient. *Pediatr Transplant,* 5**,** 60-3.

GARCIA, R. C., LEONI, P. & ALLISON, A. C. 1977. Control of phosphoribosylpyrophosphate synthesis in human lymphocytes. *Biochemical and Biophysical Research Communications,* 77**,** 1067-73.

GATTINONI, L. 2005. Removal of homeostatic cytokine sinks by lymphodepletion enhances the efficacy of adoptively transferred tumor-specific CD8+ T cells. *Journal of Experimental Medicine,* 202**,** 907-912.

GELFAND, E. W., FOX, I. H., STUCKEY, M. & DOSCH, H. M. 1978. Normal B-lymphocyte function in patients with Lesch-Nyhan syndrome and HGPRT deficiency. *Clin Exp Immunol,* 31**,** 205-8.

GERDEMANN, U., KEIRNAN, J. M., KATARI, U. L., YANAGISAWA, R., CHRISTIN, A. S., HUYE, L. E., PERNA, S. K., ENNAMURI, S., GOTTSCHALK, S., BRENNER, M. K., HESLOP, H. E., ROONEY, C. M. & LEEN, A. M. 2012. Rapidly generated multivirus-specific cytotoxic T lymphocytes for the prophylaxis and treatment of viral infections. *Molecular Therapy,* 20**,** 1622-32.

GERDEMANN, U., KEUKENS, L., KEIRNAN, J. M., KATARI, U. L., NGUYEN, C. T., DE PAGTER, A. P., RAMOS, C. A., KENNEDY-NASSER, A., GOTTSCHALK, S. M., HESLOP, H. E., BRENNER, M. K., ROONEY, C. M. & LEEN, A. M. 2013. Immunotherapeutic strategies to prevent and treat human herpesvirus 6 reactivation after allogeneic stem cell transplantation. *Blood,* 121**,** 207-18.

GIBLETT, E. R., ANDERSON, J. E., COHEN, F., POLLARA, B. & MEUWISSEN, H. J. 1972. Adenosine-deaminase deficiency in two patients with severely impaired cellular immunity. *Lancet,* 2**,** 1067-9.

GOLDRATH, A. W. & BEVAN, M. J. 1999. Selecting and maintaining a diverse T-cell repertoire. *Nature,* 402**,** 255-62.

GOLGHER, D., JONES, E., POWRIE, F., ELLIOTT, T. & GALLIMORE, A. 2002. Depletion of CD25+ regulatory cells uncovers immune responses to shared murine tumor rejection antigens. *European Journal of Immunology,* 32**,** 3267-75.

GORER, P. A. 1950. Studies in antibody response of mice to tumour inoculation. *Br J Cancer,* 4**,** 372-9.

GOSIO 1896. Richerche bacteriologiche e chimiche sulle alterazioni del mais. *Riv Igiene e Sanita Pubblica,* 7**,** 825-868.

GREEN, D. R., DROIN, N. & PINKOSKI, M. 2003. Activation-induced cell death in T cells. *Immunological Reviews,* 193**,** 70-81.

GRIFFIOEN, M., VAN EGMOND, E. H., KESTER, M. G., WILLEMZE, R., FALKENBURG, J. H. & HEEMSKERK, M. H. 2009. Retroviral transfer of human CD20 as a suicide gene for adoptive T-cell therapy. *Haematologica,* 94**,** 1316-20.

GRUPP, S. A., KALOS, M., BARRETT, D., APLENC, R., PORTER, D. L., RHEINGOLD, S. R., TEACHEY, D. T., CHEW, A., HAUCK, B., WRIGHT, J. F., MILONE, M. C., LEVINE, B. L. & JUNE, C. H. 2013. Chimeric Antigen Receptor-Modified T Cells for Acute Lymphoid Leukemia. *New England Journal of Medicine*.

GUERRA, G., CIANCIO, G., GAYNOR, J. J., ZARAK, A., BROWN, R., HANSON, L., SAGESHIMA, J., ROTH, D., CHEN, L., KUPIN, W., TUEROS, L., RUIZ, P., LIVINGSTONE, A. S. & BURKE, G. W., 3RD 2011. Randomized trial of immunosuppressive regimens in renal transplantation. *Journal of the American Society of Nephrology,* 22**,** 1758-68.

GUO, H., QIAO, Z., SU, L., ZHU, L., WANG, H. & MA, L. 2003. Analysis of immune reconstitution in adults undergoing non-myeloablative allogeneic peripheral blood stem cell transplantation. *Haematologica,* 88**,** 833-5.

GUO, Z. S., PARIMI, V., O'MALLEY, M. E., THIRUNAVUKARASU, P., SATHAIAH, M., AUSTIN, F. & BARTLETT, D. L. 2010. The combination of immunosuppression and carrier cells significantly enhances the efficacy of oncolytic poxvirus in the pre-immunized host. *Gene Therapy,* 17**,** 1465-1475.

GUSTAFSSON, A., LEVITSKY, V., ZOU, J. Z., FRISAN, T., DALIANIS, T., LJUNGMAN, P., RINGDEN, O., WINIARSKI, J., ERNBERG, I. & MASUCCI, M. G. 2000. Epstein-Barr virus (EBV) load in bone marrow transplant recipients at risk to develop posttransplant lymphoproliferative disease: prophylactic infusion of EBV-specific cytotoxic T cells. *Blood,* 95**,** 807-14.

HALE, G. A., PULE, M., AMROLIA, P. J., WEISS, H., BOLLARD, C. M., ROCHESTER, R., BRENNER, M. K., ROONEY, C. M. & HESLOP, H. E. 2008. Long-term follow-up of administration of Donor-derived EBV-specific CTLs to prevent and treat EBV Lymphoma after Hemopoietic Stem Cell Transplant *Biology of Blood and Marrow Transplantation,* 14**,** S3.

HALLORAN, P., MATHEW, T., TOMLANOVICH, S., GROTH, C., HOOFTMAN, L. & BARKER, C. 1997. Mycophenolate mofetil in renal allograft recipients: a pooled efficacy analysis of three randomized, double-blind, clinical studies in prevention of rejection. The International Mycophenolate Mofetil Renal Transplant Study Groups. *Transplantation,* 63**,** 39-47.

HAMOUR, I. M., LYSTER, H. S., BURKE, M. M., ROSE, M. L. & BANNER, N. R. 2007. Mycophenolate mofetil may allow cyclosporine and steroid sparing in de novo heart transplant patients. *Transplantation,* 83**,** 570-6.

HANAWAY, M. J., WOODLE, E. S., MULGAONKAR, S., PEDDI, V. R., KAUFMAN, D. B., FIRST, M. R., CROY, R., HOLMAN, J. & GROUP, I. S. 2011. Alemtuzumab induction in renal transplantation. *N Engl J Med,* 364**,** 1909-19.

HAO, L., LAFFERTY, K. J., ALLISON, A. C. & EUGUI, E. M. 1990. RS-61443 allows islet allografting and specific tolerance induction in adult mice. *Transplantation Proceedings,* 22**,** 876-9.

HAO, W. J., ZONG, H. T., CUI, Y. S. & ZHANG, Y. 2012. The efficacy and safety of alemtuzumab and daclizumab versus antithymocyte globulin during organ transplantation: a meta-analysis. *Transplant Proc,* 44**,** 2955-60.

HAQUE, T., AMLOT, P. L., HELLING, N., THOMAS, J. A., SWENY, P., ROLLES, K., BURROUGHS, A. K., PRENTICE, H. G. & CRAWFORD, D. H. 1998. Reconstitution of EBV-specific T cell immunity in solid organ transplant recipients. *Journal of Immunology,* 160**,** 6204-6209.

HAQUE, T., WILKIE, G. M., JONES, M. M., HIGGINS, C. D., URQUHART, G., WINGATE, P., BURNS, D., MCAULAY, K., TURNER, M., BELLAMY, C., AMLOT, P. L., KELLY, D., MACGILCHRIST, A., GANDHI, M. K., SWERDLOW, A. J. & CRAWFORD, D. H. 2007. Allogeneic cytotoxic T-cell therapy for EBV-positive posttransplantation lymphoproliferative disease: results of a phase 2 multicenter clinical trial. *Blood,* 110**,** 1123-31.

HARRIS, W. W. A. G. 1943. Investigation into the production of bacteriostatic substances by fungi. Preliminary examination of a second 100 fungal species. *British Journal of Experimental Pathology,* 24.

HART, D. P., XUE, S.-A., THOMAS, S., CESCO-GASPERE, M., TRANTER, A., WILLCOX, B., LEE, S. P., STEVEN, N., MORRIS, E. C. & STAUSS, H. J. 2008. Retroviral transfer of a dominant TCR prevents surface expression of a large proportion of the endogenous TCR repertoire in human T cells. *Gene Therapy,* 15**,** 625-631.

HEDSTROM, L., CHEUNG, K. S. & WANG, C. C. 1990. A novel mechanism of mycophenolic acid resistance in the protozoan parasite Tritrichomonas foetus. *Biochemical Pharmacology,* 39**,** 151-60.

HEDSTROM, L. & GAN, L. 2006. IMP dehydrogenase: structural schizophrenia and an unusual base. *Current Opinion in Chemical Biology,* 10**,** 520-5.

HESLOP, H. E., BRENNER, M. K., ROONEY, C., KRANCE, R. A., ROBERTS, W. M., ROCHESTER, R., SMITH, C. A., TURNER, V., SIXBEY, J., MOEN, R. & ET AL. 1994a. Administration of neomycin-resistance-gene-marked EBV-specific cytotoxic T lymphocytes to recipients of mismatched-related or phenotypically similar unrelated donor marrow grafts. *Human Gene Therapy,* 5**,** 381-97.

HESLOP, H. E., BRENNER, M. K. & ROONEY, C. M. 1994b. Donor T cells to treat EBV-associated lymphoma. *New England Journal of Medicine,* 331**,** 679-80.

HESLOP, H. E., SLOBOD, K. S., PULE, M. A., HALE, G. A., ROUSSEAU, A., SMITH, C. A., BOLLARD, C. M., LIU, H., WU, M. F., ROCHESTER, R. J., AMROLIA, P. J., HURWITZ, J. L., BRENNER, M. K. & ROONEY, C. M. 2010. Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. *Blood,* 115**,** 925-35.

HEYDE, E. & MORRISON, J. F. 1976. Studies on inosine monophosphate dehydrogenase. Isotope exchange at equilibrium. *Biochim Biophys Acta,* 429**,** 661-71.

HEYDE, E., NAGABHUSHANAM, A., VONARX, M. & MORRISON, J. F. 1976. Studies on inosine monophosphate dehydrogenase. Steady state kinetics. *Biochim Biophys Acta,* 429**,** 645-60.

HIGGINS, C. D., SWERDLOW, A. J., MACSWEEN, K. F., HARRISON, N., WILLIAMS, H., MCAULAY, K., THOMAS, R., REID, S., CONACHER, M., BRITTON, K. & CRAWFORD, D. H. 2007. A study of risk factors for acquisition of Epstein-Barr virus and its subtypes. *Journal of Infectious Diseases,* 195**,** 474-82.

HILL, G. R., CRAWFORD, J. M., COOKE, K. R., BRINSON, Y. S., PAN, L. & FERRARA, J. L. 1997. Total body irradiation and acute graft-versus-host disease: the role of gastrointestinal damage and inflammatory cytokines. *Blood,* 90**,** 3204-13.

HISLOP, A. D., TAYLOR, G. S., SAUCE, D. & RICKINSON, A. B. 2007. Cellular responses to viral infection in humans: lessons from Epstein-Barr virus. *Annual Review of Immunology,* 25**,** 587-617.

HIWARKAR, P., SHAW, B. E., TREDGER, J. M., BROWN, N. W., KULKARNI, S., SASO, R., EVANS, S., TRELEAVEN, J., DAVIES, F. E., ETHELL, M. E., MORGAN, G. J. & POTTER, M. N. 2011. Mycophenolic acid trough level monitoring: relevance in acute and chronic graft versus host disease and its relation with albumin. *Clinical Transplantation,* 25**,** 222-227.

HODGES, S. D., FUNG, E., MCKAY, D. J., RENAUX, B. S. & SNYDER, F. F. 1989. Increased activity, amount, and altered kinetic properties of IMP dehydrogenase from mycophenolic acid-resistant neuroblastoma cells. *Journal of Biological Chemistry,* 264**,** 18137-18141.

HOGQUIST, K. A., JAMESON, S. C., HEATH, W. R., HOWARD, J. L., BEVAN, M. J. & CARBONE, F. R. 1994. T cell receptor antagonist peptides induce positive selection. *Cell,* 76**,** 17-27.

HOSHIDA, Y., LI, T., DONG, Z., TOMITA, Y., YAMAUCHI, A., HANAI, J. & AOZASA, K. 2001. Lymphoproliferative disorders in renal transplant patients in Japan. *International Journal of Cancer,* 91**,** 869-75.

HSIEH, W. S., LEMAS, M. V. & AMBINDER, R. F. 1999. The biology of Epstein-Barr virus in post-transplant lymphoproliferative disease. *Transplant Infectious Disease,* 1**,** 204-12.

HUNT, J., BEDÁNOVÁ, H., STARLING, R. C., RABÁGO, G., BANNER, N. R., KOBASHIGAWA, J., KEOGH, A., KORMOS, R., MEHRA, M., WAHLERS, T. & NOELDEKE, J. 2007. 398: Premature termination of a prospective, open label, randomized, multicenter study of sirolimus to replace calcineurin inhibitors (CNI) in a standard care regimen of CNI, MMF and corticosteroids early after heart transplantation. *Journal of Heart and Lung Transplantation,* 26**,** S203.

HUPE, D. J., AZZOLINA, B. A. & BEHRENS, N. D. 1986. IMP dehydrogenase from the intracellular parasitic protozoan Eimeria tenella and its inhibition by mycophenolic acid. *Journal of Biological Chemistry,* 261**,** 8363-9.

HUSSEIN, M. M., MOOIJ, J. M. & ROUJOULEH, H. M. 2000. Regression of post-transplant Kaposi sarcoma after discontinuing cyclosporin and giving mycophenolate mofetil instead. *Nephrol Dial Transplant,* 15**,** 1103-4.

INTRONA, M., BARBUI, A. M., BAMBACIONI, F., CASATI, C., GAIPA, G., BORLERI, G., BERNASCONI, S., BARBUI, T., GOLAY, J., BIONDI, A. & RAMBALDI, A. 2000. Genetic modification of human T cells with CD20: a strategy to purify and lyse transduced cells with anti-CD20 antibodies. *Hum Gene Ther,* 11**,** 611-20.

IVANOVICS, G., MARJAI, E. & DOBOZY, A. 1968. The growth of purine mutants of Bacillus anthracis in the body of the mouse. *Journal of General Microbiology,* 53**,** 147-62.

IZERADJENE, K. & REVILLARD, J. P. 2001. Apoptosis of superantigen-activated T cells induced by mycophenolate mofetil treatment. *Transplantation,* 71**,** 118-125.

JACKSON, R. C., WEBER, G. & MORRIS, H. P. 1975. IMP dehydrogenase, an enzyme linked with proliferation and malignancy. *Nature,* 256**,** 331-333.

JAIN, J., ALMQUIST, S. J., FORD, P. J., SHLYAKHTER, D., WANG, Y., NIMMESGERN, E. & GERMANN, U. A. 2004. Regulation of inosine monophosphate dehydrogenase type I and type II isoforms in human lymphocytes. *Biochemical Pharmacology,* 67**,** 767-776.

JONES, E., DAHM-VICKER, M., SIMON, A. K., GREEN, A., POWRIE, F., CERUNDOLO, V. & GALLIMORE, A. 2002. Depletion of CD25+ regulatory cells results in suppression of melanoma growth and induction of autoreactivity in mice. *Cancer Immunity,* 2**,** 1.

JONNALAGADDA, M., BROWN, C. E., CHANG, W. C., OSTBERG, J. R., FORMAN, S. J. & JENSEN, M. C. 2013. Engineering human T cells for resistance to methotrexate and mycophenolate mofetil as an in vivo cell selection strategy. *PLoS One,* 8**,** e65519.

KALOS, M., LEVINE, B. L., PORTER, D. L., KATZ, S., GRUPP, S. A., BAGG, A. & JUNE, C. H. 2011. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Science Translational Medicine,* 3**,** 95ra73.

KARIM, M. Y., ALBA, P., CUADRADO, M. J., ABBS, I. C., D'CRUZ, D. P., KHAMASHTA, M. A. & HUGHES, G. R. 2002. Mycophenolate mofetil for systemic lupus erythematosus refractory to other immunosuppressive agents. *Rheumatology (Oxford),* 41**,** 876-82.

KASHLAN, O. B., SCOTT, C. P., LEAR, J. D. & COOPERMAN, B. S. 2002. A comprehensive model for the allosteric regulation of mammalian

ribonucleotide reductase. Functional consequences of ATP- and dATP-induced oligomerization of the large subunit. *Biochemistry,* 41**,** 462-74.

KASISKE, B. L., ZEIER, M. G., CHAPMAN, J. R., CRAIG, J. C., EKBERG, H., GARVEY, C. A., GREEN, M. D., JHA, V., JOSEPHSON, M. A., KIBERD, B. A., KREIS, H. A., MCDONALD, R. A., NEWMANN, J. M., OBRADOR, G. T., VINCENTI, F. G., CHEUNG, M., EARLEY, A., RAMAN, G., ABARIGA, S., WAGNER, M., BALK, E. M. & KIDNEY DISEASE: IMPROVING GLOBAL, O. 2010. KDIGO clinical practice guideline for the care of kidney transplant recipients: a summary. *Kidney Int,* 77**,** 299-311.

KEDL, R. M., REES, W. A., HILDEMAN, D. A., SCHAEFER, B., MITCHELL, T., KAPPLER, J. & MARRACK, P. 2000. T cells compete for access to antigen-bearing antigen-presenting cells. *Journal of Experimental Medicine,* 192**,** 1105-13.

KERSHAW, M. H., WESTWOOD, J. A., PARKER, L. L., WANG, G., ESHHAR, Z., MAVROUKAKIS, S. A., WHITE, D. E., WUNDERLICH, J. R., CANEVARI, S., ROGERS-FREEZER, L., CHEN, C. C., YANG, J. C., ROSENBERG, S. A. & HWU, P. 2006. A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. *Clinical Cancer Research,* 12**,** 6106-15.

KHANNA, R., BELL, S., SHERRITT, M., GALBRAITH, A., BURROWS, S. R., RAFTER, L., CLARKE, B., SLAUGHTER, R., FALK, M. C., DOUGLASS, J., WILLIAMS, T., ELLIOTT, S. L. & MOSS, D. J. 1999. Activation and adoptive transfer of Epstein-Barr virus-specific cytotoxic T cells in solid organ transplant patients with posttransplant lymphoproliferative disease. *Proceedings of the National Academy of Sciences of the United States of America,* 96**,** 10391-6.

KIEPER, W. C. 2002. Overexpression of Interleukin (IL)-7 Leads to IL-15-independent Generation of Memory Phenotype CD8+ T Cells. *Journal of Experimental Medicine,* 195**,** 1533-1539.

KIGUCHI, K., COLLART, F. R., HENNING-CHUBB, C. & HUBERMAN, E. 1990. Induction of cell differentiation in melanoma cells by inhibitors of IMP dehydrogenase: altered patterns of IMP dehydrogenase expression and activity. *Cell Growth and Differentiation,* 1**,** 259.

KNIGHT, S. R., RUSSELL, N. K., BARCENA, L. & MORRIS, P. J. 2009. Mycophenolate mofetil decreases acute rejection and may improve graft survival in renal transplant recipients when compared with azathioprine: a systematic review. *Transplantation,* 87**,** 785-94.

KOBASHIGAWA, J., MILLER, L., RENLUND, D., MENTZER, R., ALDERMAN, E., BOURGE, R., COSTANZO, M., EISEN, H., DUREAU, G., RATKOVEC, R., HUMMEL, M., IPE, D., JOHNSON, J., KEOGH, A., MAMELOK, R., MANCINI, D., SMART, F. & VALANTINE, H. 1998. A randomized active-controlled trial of mycophenolate mofetil in heart transplant recipients. Mycophenolate Mofetil Investigators. *Transplantation,* 66**,** 507-15.

KOCHENDERFER, J. N., DUDLEY, M. E., FELDMAN, S. A., WILSON, W. H., SPANER, D. E., MARIC, I., STETLER-STEVENSON, M., PHAN, G. Q., HUGHES, M. S., SHERRY, R. M., YANG, J. C., KAMMULA, U. S., DEVILLIER, L., CARPENTER, R., NATHAN, D. A., MORGAN, R. A., LAURENCOT, C. & ROSENBERG, S. A. 2012. B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric-antigen-receptor-transduced T cells. *Blood,* 119**,** 2709-20.

KOCHENDERFER, J. N., FELDMAN, S. A., ZHAO, Y., XU, H., BLACK, M. A., MORGAN, R. A., WILSON, W. H. & ROSENBERG, S. A. 2009. Construction and preclinical evaluation of an anti-CD19 chimeric antigen receptor. *Journal of Immunotherapy,* 32**,** 689-702.

KOCHENDERFER, J. N., WILSON, W. H., JANIK, J. E., DUDLEY, M. E., STETLER-STEVENSON, M., FELDMAN, S. A., MARIC, I., RAFFELD, M., NATHAN, D. A., LANIER, B. J., MORGAN, R. A. & ROSENBERG, S. A. 2010a. Eradication of B-lineage cells and regression of lymphoma in a patient treated with autologous T cells genetically engineered to recognize CD19. *Blood,* 116**,** 4099-102.

KOCHENDERFER, J. N., YU, Z., FRASHERI, D., RESTIFO, N. P. & ROSENBERG, S. A. 2010b. Adoptive transfer of syngeneic T cells transduced with a chimeric antigen receptor that recognizes murine CD19 can eradicate lymphoma and normal B cells. *Blood,* 116**,** 3875-86.

KOEHL, G. E., WAGNER, F., STOELTZING, O., LANG, S. A., STEINBAUER, M., SCHLITT, H. J. & GEISSLER, E. K. 2007. Mycophenolate Mofetil Inhibits Tumor Growth and Angiogenesis In Vitro but Has Variable Antitumor Effects In Vivo, Possibly Related to Bioavailability. *Transplantation,* 83**,** 607-614.

KOHLER, G. & MILSTEIN, C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature,* 256**,** 495-7.

KOHLER, G. A., WHITE, T. C. & AGABIAN, N. 1997. Overexpression of a cloned IMP dehydrogenase gene of Candida albicans confers resistance to the specific inhibitor mycophenolic acid. *Journal of Bacteriology,* 179**,** 2331-8.

KOLB, H. J., MITTERMULLER, J., CLEMM, C., HOLLER, E., LEDDEROSE, G., BREHM, G., HEIM, M. & WILMANNS, W. 1990. Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients. *Blood,* 76**,** 2462-5.

KOOPMAN, G., REUTELINGSPERGER, C. P., KUIJTEN, G. A., KEEHNEN, R. M., PALS, S. T. & VAN OERS, M. H. 1994. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood,* 84**,** 1415-20.

KOZHEVNIKOVA, E. N., VAN DER KNAAP, J. A., PINDYURIN, A. V., OZGUR, Z., VAN IJCKEN, W. F., MOSHKIN, Y. M. & VERRIJZER, C. P. 2012.

Metabolic enzyme IMPDH is also a transcription factor regulated by cellular state. *Molecular Cell,* 47**,** 133-9.

KU, C. C. 2000. Control of Homeostasis of CD8+ Memory T Cells by Opposing Cytokines. *Science,* 288**,** 675-678.

KUPPERS, R. 2003. B cells under influence: transformation of B cells by Epstein-Barr virus. *Nature Reviews: Immunology,* 3**,** 801-12.

KUYPERS, D. R., DE JONGE, H., NAESENS, M., DE LOOR, H., HALEWIJCK, E., DEKENS, M. & VANRENTERGHEM, Y. 2008. Current target ranges of mycophenolic acid exposure and drug-related adverse events: a 5-year, open-label, prospective, clinical follow-up study in renal allograft recipients. *Clin Ther,* 30**,** 673-83.

KUYPERS, D. R., LE MEUR, Y., CANTAROVICH, M., TREDGER, M. J., TETT, S. E., CATTANEO, D., TONSHOFF, B., HOLT, D. W., CHAPMAN, J. & GELDER, T. 2010. Consensus report on therapeutic drug monitoring of mycophenolic acid in solid organ transplantation. *Clin J Am Soc Nephrol,* 5**,** 341-58.

LE GUELLEC, C., BOURGOIN, H., BUCHLER, M., LE MEUR, Y., LEBRANCHU, Y., MARQUET, P. & PAINTAUD, G. 2004. Population pharmacokinetics and Bayesian estimation of mycophenolic acid concentrations in stable renal transplant patients. *Clin Pharmacokinet,* 43**,** 253-66.

LE MEUR, Y., BÜCHLER, M., THIERRY, A., CAILLARD, S., VILLEMAIN, F., LAVAUD, S., ETIENNE, I., WESTEEL, P. F., DE LIGNY, B. H., ROSTAING, L., THERVET, E., SZELAG, J. C., RÉROLLE, J. P., ROUSSEAU, A., TOUCHARD, G. & MARQUET, P. 2007. Individualized Mycophenolate Mofetil Dosing Based on Drug Exposure Significantly Improves Patient Outcomes After Renal Transplantation. *American Journal of Transplantation,* 7**,** 2496-2503.

LEBLOND, V., SUTTON, L., DORENT, R., DAVI, F., BITKER, M. O., GABARRE, J., CHARLOTTE, F., GHOUSSOUB, J. J., FOURCADE, C., FISCHER, A. & ET AL. 1995. Lymphoproliferative disorders after organ transplantation: a report of 24 cases observed in a single center. *Journal of Clinical Oncology,* 13**,** 961-8.

LEE, W. A., GU, L., MIKSZTAL, A. R., CHU, N., LEUNG, K. & NELSON, P. H. 1990. Bioavailability improvement of mycophenolic acid through amino ester derivatization. *Pharmacological Research,* 7**,** 161-6.

LEEN, A. M., SILI, U., SAVOLDO, B., JEWELL, A. M., PIEDRA, P. A., BRENNER, M. K. & ROONEY, C. M. 2004. Fiber-modified adenoviruses generate subgroup cross-reactive, adenovirus-specific cytotoxic T lymphocytes for therapeutic applications. *Blood,* 103**,** 1011-9.

LETOURNEUR, F. & MALISSEN, B. 1989. Derivation of a T cell hybridoma variant deprived of functional T cell receptor alpha and beta chain

transcripts reveals a nonfunctional alpha-mRNA of BW5147 origin. *Eur J Immunol,* 19**,** 2269-74.

LI, H., MAGER, D. E., SANDMAIER, B. M., MALONEY, D. G., BEMER, M. J. & MCCUNE, J. S. 2013. Population pharmacokinetics and dose optimization of mycophenolic acid in HCT recipients receiving oral mycophenolate mofetil. *J Clin Pharmacol,* 53**,** 393-402.

LI, H., MAGER, D. E., SANDMAIER, B. M., STORER, B. E., BOECKH, M. J., BEMER, M. J., PHILLIPS, B. R., RISLER, L. J. & MCCUNE, J. S. 2014. Pharmacokinetic and pharmacodynamic analysis of inosine monophosphate dehydrogenase activity in hematopoietic cell transplantation recipients treated with mycophenolate mofetil. *Biol Blood Marrow Transplant,* 20**,** 1121-9.

LIGHTFOOT, T. & SNYDER, F. F. 1994. Gene amplification and dual point mutations of mouse IMP dehydrogenase associated with cellular resistance to mycophenolic acid. *Biochimica et biophysica acta,* 1217**,** 156-162.

LINK, J. & STRAUB, K. 1996. Trapping of an IMP dehydrogenase-substrate covalent intermediate by mycophenolic acid. *Journal of the American Chemical Society,* 118**,** 2091-2092.

LINNEMANN, C., HEEMSKERK, B., KVISTBORG, P., KLUIN, R. J., BOLOTIN, D. A., CHEN, X., BRESSER, K., NIEUWLAND, M., SCHOTTE, R., MICHELS, S., GOMEZ-EERLAND, R., JAHN, L., HOMBRINK, P., LEGRAND, N., SHU, C. J., MAMEDOV, I. Z., VELDS, A., BLANK, C. U., HAANEN, J. B., TURCHANINOVA, M. A., KERKHOVEN, R. M., SPITS, H., HADRUP, S. R., HEEMSKERK, M. H., BLANKENSTEIN, T., CHUDAKOV, D. M., BENDLE, G. M. & SCHUMACHER, T. N. 2013. High-throughput identification of antigen-specific TCRs by TCR gene capture. *Nature Medicine,* 19**,** 1534-41.

LIU, Y., BOHN, S. A. & SHERLEY, J. L. 1998. Inosine-5'-monophosphate dehydrogenase is a rate-determining factor for p53-dependent growth regulation. *Molecular biology of the cell,* 9**,** 15-28.

LJUNGGREN, H. G., STAM, N. J., OHLÉN, C., NEEFJES, J. J., HÖGLUND, P., HEEMELS, M. T., BASTIN, J., SCHUMACHER, T. N., TOWNSEND, A. & KÄRRE, K. 1990. Empty MHC class I molecules come out in the cold. *Nature,* 346**,** 476-480.

LJUNGMAN, P., HAKKI, M. & BOECKH, M. 2011. Cytomegalovirus in hematopoietic stem cell transplant recipients. *Hematol Oncol Clin North Am,* 25**,** 151-69.

LOOK, M., STERN, E., WANG, Q. A., DIPLACIDO, L. D., KASHGARIAN, M., CRAFT, J. & FAHMY, T. M. 2013. Nanogel-based delivery of mycophenolic acid ameliorates systemic lupus erythematosus in mice. *Journal of Clinical Investigation*.

MACKALL, C. L., HAKIM, F. T. & GRESS, R. E. 1997. Restoration of T-cell homeostasis after T-cell depletion. *Seminars in Immunology,* 9**,** 339-46.

MAKARA, G. M., KESERU, G. M., KAJTAR-PEREDY, M. & ANDERSON, W. K. 1996. Nuclear magnetic resonance and molecular modeling study on mycophenolic acid: implications for binding to inosine monophosphate dehydrogenase. *Journal of Medicinal Chemistry,* 39**,** 1236-42.

MARIS, M. B., SANDMAIER, B. M., STORER, B. E., STUART, M., AGURA, E., HEGENBART, U., PULSIPHER, M. A., MAZIARZ, R. T., BRUNO, B., MCSWEENEY, P., MALONEY, D. G., NIEDERWIESER, D., BLUME, K. & STORB, R. 2004. TID compared to BID mycophenolate mofetil (MMF) improves donor chimerism and engraftment rates without increasing postgrafting toxicities after unrelated peripheral blood stem cell (PBSC) transplantation (HCT) with nonmyeloablative *Biology of Blood and Marrow Transplantation,* 10**,** 35-36.

MARKS-KONCZALIK, J., DUBOIS, S., LOSI, J. M., SABZEVARI, H., YAMADA, N., FEIGENBAUM, L., WALDMANN, T. A. & TAGAYA, Y. 2000. IL-2-induced activation-induced cell death is inhibited in IL-15 transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America,* 97**,** 11445-50.

MARRACK, P., BENDER, J., HILDEMAN, D., JORDAN, M., MITCHELL, T., MURAKAMI, M., SAKAMOTO, A., SCHAEFER, B. C., SWANSON, B. & KAPPLER, J. 2000. Homeostasis of alpha beta TCR+ T cells. *Nature Immunology,* 1**,** 107-11.

MATAS, A. J., SMITH, J. M., SKEANS, M. A., LAMB, K. E., GUSTAFSON, S. K., SAMANA, C. J., STEWART, D. E., SNYDER, J. J., ISRANI, A. K. & KASISKE, B. L. 2013. OPTN/SRTR 2011 Annual Data Report: kidney. *American Journal of Transplantation,* 13 Suppl 1**,** 11-46.

MCFARLAND, W. C. & STOCKER, B. A. 1987. Effect of different purine auxotrophic mutations on mouse-virulence of a Vi-positive strain of Salmonella dublin and of two strains of Salmonella typhimurium. *Microbial Pathogenesis,* 3**,** 129-41.

MCLEAN, J. E., HAMAGUCHI, N., BELENKY, P., MORTIMER, S. E., STANTON, M. & HEDSTROM, L. 2004. Inosine 5'-monophosphate dehydrogenase binds nucleic acids in vitro and in vivo. *Biochemical Journal,* 379**,** 243-51.

MEHLING, A., GRABBE, S., VOSKORT, M., SCHWARZ, T., LUGER, T. A. & BEISSERT, S. 2000. Mycophenolate mofetil impairs the maturation and function of murine dendritic cells. *The Journal of immunology,* 165**,** 2374-2381.

MERLO, A., TURRINI, R., DOLCETTI, R., ZANOVELLO, P., AMADORI, A. & ROSATO, A. 2008. Adoptive cell therapy against EBV-related malignancies: a survey of clinical results. *Expert Opinion on Biological Therapy,* 8**,** 1265-94.

MESHKINI, A., YAZDANPARAST, R. & NOURI, K. 2011. Intracellular GTP level determines cell's fate toward differentiation and apoptosis. *Toxicology and Applied Pharmacology,* 253**,** 188-196.

MESSINA, E., GAZZANIGA, P., MICHELI, V., BARILE, L., LUPI, F., AGLIANO, A. M. & GIACOMELLO, A. 2004. Low levels of mycophenolic acid induce differentiation of human neuroblastoma cell lines. *International Journal of Cancer,* 112**,** 352-4.

MESSINA, E., MICHELI, V. & GIACOMELLO, A. 2005. Guanine nucleotide depletion induces differentiation and aberrant neurite outgrowth in human dopaminergic neuroblastoma lines: a model for basal ganglia dysfunction in Lesch-Nyhan disease. *Neuroscience Letters,* 375**,** 97-100.

MICHEL, L., VUKUSIC, S., DE SEZE, J., DUCRAY, F., ONGAGNA, J. C., LEFRERE, F., JACQ-FOUCHER, M., CONFAVREUX, C., WIERTLEWSKI, S. & LAPLAUD, D. A. 2013. Mycophenolate mofetil in multiple sclerosis: a multicentre retrospective study on 344 patients. *Journal of Neurology, Neurosurgery and Psychiatry*.

MILLER-KITTRELL, M. & SPARER, T. E. 2009. Feeling manipulated: cytomegalovirus immune manipulation. *Virol J,* 6**,** 4.

MITSUI, A. & SUZUKI, S. 1969. Immunosuppressive effect of mycophenolic acid. *Journal of Antibiotics,* 22**,** 358-63.

MODRY, D. L., OYER, P. E., JAMIESON, S. W., STINSON, E. B., BALDWIN, J. C., REITZ, B. A., DAWKINS, K. D., MCGREGOR, C. G., HUNT, S. A., MORAN, M. & ET AL. 1985. Cyclosporine in heart and heart-lung transplantation. *Can J Surg,* 28**,** 274-80, 282.

MONCADA, S., PALMER, R. M. & HIGGS, E. A. 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacological Reviews,* 43**,** 109-42.

MOORE, M. W., CARBONE, F. R. & BEVAN, M. J. 1988. Introduction of soluble protein into the class I pathway of antigen processing and presentation. *Cell,* 54**,** 777-785.

MOORE, R. A. & DERRY, S. 2006. Systematic review and meta-analysis of randomised trials and cohort studies of mycophenolate mofetil in lupus nephritis. *Arthritis Research & Therapy,* 8**,** R182.

MORGAN, R. A., DUDLEY, M. E., WUNDERLICH, J. R., HUGHES, M. S., YANG, J. C., SHERRY, R. M., ROYAL, R. E., TOPALIAN, S. L., KAMMULA, U. S., RESTIFO, N. P., ZHENG, Z., NAHVI, A., DE VRIES, C. R., ROGERS-FREEZER, L. J., MAVROUKAKIS, S. A. & ROSENBERG, S. A. 2006. Cancer Regression in Patients After Transfer of Genetically Engineered Lymphocytes. *Science,* 314**,** 126-129.

MORRIS, E. C., TSALLIOS, A., BENDLE, G. M., XUE, S. & STAUSS, H. J. 2005. A critical role of T cell antigen receptor-transduced MHC class I-restricted helper T cells in tumor protection. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 7934.

MORRIS, R. E., HOYT, E. G., MURPHY, M. P., EUGUI, E. M. & ALLISON, A. C. 1990. Mycophenolic acid morpholinoethylester (RS-61443) is a new immunosuppressant that prevents and halts heart allograft rejection by

selective inhibition of T- and B-cell purine synthesis. *Transplantation Proceedings,* 22**,** 1659-62.

MORRIS, R. E., WANG, J., BLUM, J. R., FLAVIN, T., MURPHY, M. P., ALMQUIST, S. J., CHU, N., TAM, Y. L., KALOOSTIAN, M., ALLISON, A. C. & ET AL. 1991. Immunosuppressive effects of the morpholinoethyl ester of mycophenolic acid (RS-61443) in rat and nonhuman primate recipients of heart allografts. *Transplantation Proceedings,* 23**,** 19-25.

MORRISON, H. G., MCARTHUR, A. G., GILLIN, F. D., ALEY, S. B., ADAM, R. D., OLSEN, G. J., BEST, A. A., CANDE, W. Z., CHEN, F., CIPRIANO, M. J., DAVIDS, B. J., DAWSON, S. C., ELMENDORF, H. G., HEHL, A. B., HOLDER, M. E., HUSE, S. M., KIM, U. U., LASEK-NESSELQUIST, E., MANNING, G., NIGAM, A., NIXON, J. E., PALM, D., PASSAMANECK, N. E., PRABHU, A., REICH, C. I., REINER, D. S., SAMUELSON, J., SVARD, S. G. & SOGIN, M. L. 2007. Genomic minimalism in the early diverging intestinal parasite Giardia lamblia. *Science,* 317**,** 1921-6.

MORTIMER, S. E. & HEDSTROM, L. 2005. Autosomal dominant retinitis pigmentosa mutations in inosine 5'-monophosphate dehydrogenase type I disrupt nucleic acid binding. *Biochemical Journal,* 390**,** 41-7.

MORTIMER, S. E., XU, D., MCGREW, D., HAMAGUCHI, N., LIM, H. C., BOWNE, S. J., DAIGER, S. P. & HEDSTROM, L. 2008. IMP dehydrogenase type 1 associates with polyribosomes translating rhodopsin mRNA. *Journal of Biological Chemistry,* 283**,** 36354-60.

MUELLER, K., VON MASSENHAUSEN, A., AICHELE, U., STARCK, L., LEISEGANG, M., UCKERT, W. & PIRCHER, H. 2012. Protective capacity of virus-specific T cell receptor-transduced CD8 T cells in vivo. *Journal of Virology,* 86**,** 10866-9.

MURRAY, J. E., MERRILL, J. P., HARRISON, J. H., WILSON, R. E. & DAMMIN, G. J. 1963. Prolonged survival of human-kidney homografts by immunosuppressive drug therapy. *N Engl J Med,* 268**,** 1315-23.

MUTI, G., KLERSY, C., BALDANTI, F., GRANATA, S., ORESTE, P., PEZZETTI, L., GATTI, M., GARGANTINI, L., CARAMELLA, M., MANCINI, V., GERNA, G., MORRA, E. & CO-OPERATIVE STUDY GROUP ON, P. 2003. Epstein-Barr virus (EBV) load and interleukin-10 in EBV-positive and EBV-negative post-transplant lymphoproliferative disorders. *British Journal of Haematology,* 122**,** 927-33.

NADLER, L. M., ANDERSON, K. C., MARTI, G., BATES, M., PARK, E., DALEY, J. F. & SCHLOSSMAN, S. F. 1983. B4, a human B lymphocyte-associated antigen expressed on normal, mitogen-activated, and malignant B lymphocytes. *Journal of Immunology,* 131**,** 244-50.

NAGAI, M., NATSUMEDA, Y., KONNO, Y., HOFFMAN, R., IRINO, S. & WEBER, G. 1991. Selective up-regulation of type II inosine 5'-monophosphate dehydrogenase messenger RNA expression in human leukemias. *Cancer Research,* 51**,** 3886-3890.

NAGAI, M., NATSUMEDA, Y. & WEBER, G. 1992. Proliferation-linked regulation of type II IMP dehydrogenase gene in human normal lymphocytes and HL-60 leukemic cells. *Cancer Research,* 52**,** 258-261.

NAGY, S. E., ANDERSSON, J. P. & ANDERSSON, U. G. 1993. Effect of mycophenolate mofetil (RS-61443) on cytokine production: inhibition of superantigen-induced cytokines. *Immunopharmacology,* 26**,** 11-20.

NAKAJIMA, K., OCHIAI, T., NAGATA, M., SUZUKI, T., GUNJI, Y., ASANO, T., SHIMADA, H. & ISONO, K. 1993. Effects of triple therapy of cyclosporine, FK 506, and RS-61443 on allogeneic small bowel transplantation in dogs. *Transplantation Proceedings,* 25**,** 595-6.

NATALI, P. G., BIGOTTI, A., NICOTRA, M. R., VIORA, M., MANFREDI, D. & FERRONE, S. 1984. Distribution of human Class I (HLA-A,B,C) histocompatibility antigens in normal and malignant tissues of nonlymphoid origin. *Cancer Research,* 44**,** 4679-87.

NATSUMEDA, Y., OHNO, S., KAWASAKI, H., KONNO, Y., WEBER, G. & SUZUKI, K. 1990. Two distinct cDNAs for human IMP dehydrogenase. *Journal of Biological Chemistry,* 265**,** 5292-5295.

NEUMANN, I., FUHRMANN, H., FANG, I.-F., JAEGER, A., BAYER, P. & KOVARIK, J. 2008. Association between mycophenolic acid 12-h trough levels and clinical endpoints in patients with autoimmune disease on mycophenolate mofetil. *Nephrology Dialysis Transplantation,* 23**,** 3514-3520.

NEWELL, K. A., ALONSO, E. M., WHITINGTON, P. F., BRUCE, D. S., MILLIS, J. M., PIPER, J. B., WOODLE, E. S., KELLY, S. M., KOEPPEN, H., HART, J., RUBIN, C. M. & THISTLETHWAITE, J. R., JR. 1996. Posttransplant lymphoproliferative disease in pediatric liver transplantation. Interplay between primary Epstein-Barr virus infection and immunosuppression. *Transplantation,* 62**,** 370-5.

NEYTS, J., ANDREI, G. & DE CLERCQ, E. 1998. The novel immunosuppressive agent mycophenolate mofetil markedly potentiates the antiherpesvirus activities of acyclovir, ganciclovir, and penciclovir in vitro and in vivo. *Antimicrob Agents Chemother,* 42**,** 216-22.

NIMMESGERN, E., BLACK, J., FUTER, O., FULGHUM, J. R., CHAMBERS, S. P., BRUMMEL, C. L., RAYBUCK, S. A. & SINTCHAK, M. D. 1999. Biochemical analysis of the modular enzyme inosine 5'-monophosphate dehydrogenase. *Protein Expression and Purification,* 17**,** 282-9.

NORIEGA, F. R., LOSONSKY, G., WANG, J. Y., FORMAL, S. B. & LEVINE, M. M. 1996. Further characterization of delta aroA delta virG Shigella flexneri 2a strain CVD 1203 as a mucosal Shigella vaccine and as a live-vector vaccine for delivering antigens of enterotoxigenic Escherichia coli. *Infection and Immunity,* 64**,** 23-7.

NOTO, T., SAWADA, M., ANDO, K. & KOYAMA, K. 1969. Some biological properties of mycophenolic acid. *Journal of Antibiotics,* 22**,** 165-9.

NOWAK, I. & SHAW, L. M. 1995. Mycophenolic acid binding to human serum albumin: characterization and relation to pharmacodynamics. *Clinical Chemistry,* 41**,** 1011-7.

OAKS, T. E., WANNENBERG, T., CLOSE, S. A., TUTTLE, L. E. & KON, N. D. 2001. Steroid-free maintenance immunosuppression after heart transplantation. *Annals of Thoracic Surgery,* 72**,** 102-6.

OHATA, K., ESPINOZA, J. L., LU, X., KONDO, Y. & NAKAO, S. 2011. Mycophenolic Acid Inhibits Natural Killer Cell Proliferation and Cytotoxic Function: A Possible Disadvantage of Including Mycophenolate Mofetil in the Graft-Versus-Host Disease Prophylaxis Regimen. *Biology of Blood and Marrow Transplantation,* 17**,** 205-213.

OJO, A. O., MEIER-KRIESCHE, H. U., HANSON, J. A., LEICHTMAN, A. B., CIBRIK, D., MAGEE, J. C., WOLFE, R. A., AGODOA, L. Y. & KAPLAN, B. 2000. Mycophenolate mofetil reduces late renal allograft loss independent of acute rejection. *Transplantation,* 69**,** 2405-9.

OKAMURA, A., SHIMOYAMA, M., ISHII, S., WAKAHASHI, K., ASADA, N., KAWANO, H., KAWAMORI, Y., NISHIKAWA, S., MINAGAWA, K., KATAYAMA, Y. & MATSUI, T. 2011. Delayed neutrophil engraftment in cord blood transplantation with intensive administration of mycophenolate mofetil for GVHD prophylaxis. *Bone Marrow Transplant,* 46**,** 148-9.

OPELZ, G. & DOHLER, B. 2003. Lymphomas After Solid Organ Transplantation:A Collaborative Transplant Study Report. *American Journal of Transplantation,* 4**,** 222-230.

OREMUS, M., ZEIDLER, J., ENSOM, M. H., MATSUDA-ABEDINI, M., BALION, C., BOOKER, L., ARCHER, C. & RAINA, P. 2008. Utility of monitoring mycophenolic acid in solid organ transplant patients. *Evidence Report/Technology Assessment.* 2008/05/07 ed.

ORENTAS, R. J., ROSKOPF, S. J., NOLAN, G. P. & NISHIMURA, M. I. 2001. Retroviral transduction of a T cell receptor specific for an Epstein-Barr virus-encoded peptide. *Clinical Immunology,* 98**,** 220-8.

OUYANG, Q., WAGNER, W. M., WIKBY, A., WALTER, S., AUBERT, G., DODI, A. I., TRAVERS, P. & PAWELEC, G. 2003. Large numbers of dysfunctional CD8+ T lymphocytes bearing receptors for a single dominant CMV epitope in the very old. *J Clin Immunol,* 23**,** 247-57.

PADALKO, E., VERBEKEN, E., MATTHYS, P., AERTS, J. L., DE CLERCQ, E. & NEYTS, J. 2003. Mycophenolate mofetil inhibits the development of Coxsackie B3-virus-induced myocarditis in mice. *BMC Microbiol,* 3**,** 25.

PALMER, E. 2003. Negative selection--clearing out the bad apples from the T-cell repertoire. *Nature Reviews: Immunology,* 3**,** 383-91.

PAPADOPOULOS, E. B., LADANYI, M., EMANUEL, D., MACKINNON, S., BOULAD, F., CARABASI, M. H., CASTRO-MALASPINA, H., CHILDS, B. H., GILLIO, A. P., SMALL, T. N. & ET AL. 1994. Infusions of donor

leukocytes to treat Epstein-Barr virus-associated lymphoproliferative disorders after allogeneic bone marrow transplantation. *New England Journal of Medicine,* 330**,** 1185-91.

PEGGS, K. S., VERFUERTH, S., PIZZEY, A., KHAN, N., GUIVER, M., MOSS, P. A. & MACKINNON, S. 2003. Adoptive cellular therapy for early cytomegalovirus infection after allogeneic stem-cell transplantation with virus-specific T-cell lines. *Lancet,* 362**,** 1375-7.

PERRO, M., TSANG, J., XUE, S. A., ESCORS, D., CESCO-GASPERE, M., POSPORI, C., GAO, L., HART, D., COLLINS, M., STAUSS, H. & MORRIS, E. C. 2010. Generation of multi-functional antigen-specific human T-cells by lentiviral TCR gene transfer. *Gene Therapy,* 17**,** 721-32.

PERRUCCIO, K., TOSTI, A., BURCHIELLI, E., TOPINI, F., RUGGERI, L., CAROTTI, A., CAPANNI, M., URBANI, E., MANCUSI, A., AVERSA, F., MARTELLI, M. F., ROMANI, L. & VELARDI, A. 2005. Transferring functional immune responses to pathogens after haploidentical hematopoietic transplantation. *Blood,* 106**,** 4397-406.

PIMKIN, M. & MARKHAM, G. D. 2008. The CBS subdomain of inosine 5'-monophosphate dehydrogenase regulates purine nucleotide turnover. *Molecular Microbiology,* 68**,** 342-59.

PLANTEROSE, D. N. 1969. Antiviral and cytotoxic effects of mycophenolic acid. *Journal of General and Molecular Virology,* 4**,** 629-30.

PLATZ, K. P., ECKHOFF, D. E., HULLETT, D. A. & SOLLINGER, H. W. 1991. Prolongation of dog renal allograft survival by RS-61443, a new, potent immunosuppressive agent. *Transplantation Proceedings,* 23**,** 497-8.

POIRE, X. & VAN BESIEN, K. 2011. Alemtuzumab in allogeneic hematopoetic stem cell transplantation. *Expert Opin Biol Ther,* 11**,** 1099-111.

PONCE, D. M., HARNICAR, S., HILDEN, P., DEVLIN, S., EVANS, K. L., LUBIN, M., KERNAN, N. A., PROCKOP, S. E., SCARADAVOU, A., GIRALT, S., GOLDBERG, J. D., PERALES, M. A. & BARKER, J. N. 2014. Intensified Mycophenolate Mofetil (MMF) Dosing Is Safe from the Standpoint of Engraftment and Reduces Severe Acute Graft-Versus-Host Disease (aGVHD) after Double-Unit Cord Blood Transplantation (DCBT): An Analysis of 173 Patients. *Biology of Blood and Marrow Transplantation,* 20**,** S279-S280.

POOLE, E., MCGREGOR DALLAS, S. R., COLSTON, J., JOSEPH, R. S. & SINCLAIR, J. 2011. Virally induced changes in cellular microRNAs maintain latency of human cytomegalovirus in CD34(+) progenitors. *Journal of General Virology,* 92**,** 1539-49.

PORTER, D. L., LEVINE, B. L., KALOS, M., BAGG, A. & JUNE, C. H. 2011. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *New England Journal of Medicine,* 365**,** 725-33.

PROVASI, E., GENOVESE, P., LOMBARDO, A., MAGNANI, Z., LIU, P. Q., REIK, A., CHU, V., PASCHON, D. E., ZHANG, L., KUBALL, J., CAMISA, B., BONDANZA, A., CASORATI, G., PONZONI, M., CICERI, F., BORDIGNON, C., GREENBERG, P. D., HOLMES, M. C., GREGORY, P. D., NALDINI, L. & BONINI, C. 2012. Editing T cell specificity towards leukemia by zinc finger nucleases and lentiviral gene transfer. *Nature Medicine,* 18**,** 807-15.

QUÉMÉNEUR, L., BELOEIL, L., MICHALLET, M.-C., ANGELOV, G., TOMKOWIAK, M., REVILLARD, J.-P. & MARVEL, J. 2004. Restriction of de novo nucleotide biosynthesis interferes with clonal expansion and differentiation into effector and memory CD8 T cells. *The Journal of immunology,* 173**,** 4945-4952.

RAISANEN-SOKOLOWSKI, A., MYLLARNIEMI, M. & HAYRY, P. 1994. Effect of mycophenolate mofetil on allograft arteriosclerosis (chronic rejection). *Transplantation Proceedings,* 26**,** 3225.

RAJAB, A., PELLETIER, R. P., HENRY, M. L. & FERGUSON, R. M. 2006. Excellent clinical outcomes in primary kidney transplant recipients treated with steroid-free maintenance immunosuppression. *Clinical Transplantation,* 20**,** 537-46.

RAMOS, M. A., PINERA, C., SETIEN, M. A., BUELTA, L., DE COS, M. A., DE FRANCISCO, A. L. M., MERINO, R. & ARIAS, M. 2003. Modulation of autoantibody production by mycophenolate mofetil: effects on the development of SLE in (NZBxNZW)F1 mice. *Nephrology Dialysis Transplantation,* 18**,** 878-883.

RICCIARDELLI, I., BLUNDELL, M. P., BREWIN, J., PULE, M. & AMROLIA, P. J. 2014. Towards gene therapy for EBV-associated Post-Transplant Lymphoproliferative disease: genetically modified EBV-specific Cytotoxic T Lymphocytes induce regression of autologous EBV-induced Lymphoproliferation despite immunosuppression. *Blood*.

RICHARDSON, P. D., JAMES, P. D. & RYDER, S. D. 2000. Mycophenolate mofetil for maintenance of remission in autoimmune hepatitis in patients resistant to or intolerant of azathioprine. *Journal of Hepatology,* 33**,** 371-5.

RIDDELL, S. R., WATANABE, K. S., GOODRICH, J. M., LI, C. R., AGHA, M. E. & GREENBERG, P. D. 1992. Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. *Science,* 257**,** 238-41.

RIGBY, S. M., ROUSE, T. & FIELD, E. H. 2003. Total lymphoid irradiation nonmyeloablative preconditioning enriches for IL-4-producing CD4+-TNK cells and skews differentiation of immunocompetent donor CD4+ cells. *Blood,* 101**,** 2024-32.

ROBERTS, D. M., JIANG, S. H. & CHADBAN, S. J. 2012. The treatment of acute antibody-mediated rejection in kidney transplant recipients-a systematic review. *Transplantation,* 94**,** 775-83.

ROBSON, R., CECKA, J. M., OPELZ, G., BUDDE, M. & SACKS, S. 2005. Prospective registry-based observational cohort study of the long-term risk of malignancies in renal transplant patients treated with mycophenolate mofetil. *Am J Transplant,* 5**,** 2954-60.

ROONEY, C. M., SMITH, C. A., NG, C. Y., LOFTIN, S., LI, C., KRANCE, R. A., BRENNER, M. K. & HESLOP, H. E. 1995. Use of gene-modified virus-specific T lymphocytes to control Epstein-Barr-virus-related lymphoproliferation. *Lancet,* 345**,** 9-13.

ROONEY, C. M., SMITH, C. A., NG, C. Y., LOFTIN, S. K., SIXBEY, J. W., GAN, Y., SRIVASTAVA, D. K., BOWMAN, L. C., KRANCE, R. A., BRENNER, M. K. & HESLOP, H. E. 1998. Infusion of cytotoxic T cells for the prevention and treatment of Epstein-Barr virus-induced lymphoma in allogeneic transplant recipients. *Blood,* 92**,** 1549-55.

ROSENBERG, S. A., AEBERSOLD, P., CORNETTA, K., KASID, A., MORGAN, R. A., MOEN, R., KARSON, E. M., LOTZE, M. T., YANG, J. C., TOPALIAN, S. L. & ET AL. 1990. Gene transfer into humans--immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *New England Journal of Medicine,* 323**,** 570-8.

ROSENBERG, S. A., PACKARD, B. S., AEBERSOLD, P. M., SOLOMON, D., TOPALIAN, S. L., TOY, S. T., SIMON, P., LOTZE, M. T., YANG, J. C., SEIPP, C. A. & ET AL. 1988. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. *New England Journal of Medicine,* 319**,** 1676-80.

ROSSIG, C., BAR, A., PSCHERER, S., ALTVATER, B., PULE, M., ROONEY, C. M., BRENNER, M. K., JURGENS, H. & VORMOOR, J. 2006. Target antigen expression on a professional antigen-presenting cell induces superior proliferative antitumor T-cell responses via chimeric T-cell receptors. *Journal of Immunotherapy,* 29**,** 21-31.

ROTZSCHKE, O., FALK, K., STEVANOVIC, S., JUNG, G., WALDEN, P. & RAMMENSEE, H. G. 1991. Exact prediction of a natural T cell epitope. *Eur J Immunol,* 21**,** 2891-4.

RUSSO, V., TANZARELLA, S., DALERBA, P., RIGATTI, D., ROVERE, P., VILLA, A., BORDIGNON, C. & TRAVERSARI, C. 2000. Dendritic cells acquire the MAGE-3 human tumor antigen from apoptotic cells and induce a class I-restricted T cell response. *Proceedings of the National Academy of Sciences of the United States of America,* 97**,** 2185-90.

RUUTU, T., VAN BIEZEN, A., HERTENSTEIN, B., HENSELER, A., GARDERET, L., PASSWEG, J., MOHTY, M., SUREDA, A., NIEDERWIESER, D., GRATWOHL, A. & DE WITTE, T. 2012. Prophylaxis and treatment of GVHD after allogeneic haematopoietic SCT: a survey of centre strategies by the European Group for Blood and Marrow Transplantation. *Bone Marrow Transplant,* 47**,** 1459-64.

SADELAIN, M., RIVIERE, I. & BRENTJENS, R. 2003. Targeting tumours with genetically enhanced T lymphocytes. *Nature Reviews: Cancer,* 3**,** 35-45.

SALINAS-CARMONA, M. C., PEREZ, L. I., GALAN, K. & VAZQUEZ, A. V. 2009. Immunosuppressive drugs have different effect on B lymphocyte subsets and IgM antibody production in immunized BALB/c mice. *Autoimmunity,* 42**,** 537-44.

SALIS, P., CACCAMO, C., VERZARO, R., GRUTTADAURIA, S. & ARTERO, M. 2008. The role of basiliximab in the evolving renal transplantation immunosuppression protocol. *Biologics,* 2**,** 175-88.

SALTER, R. D. & CRESSWELL, P. 1986. Impaired assembly and transport of HLA-A and -B antigens in a mutant TxB cell hybrid. *EMBO J,* 5**,** 943-9.

SALTER, R. D., HOWELL, D. N. & CRESSWELL, P. 1985. Genes regulating HLA class I antigen expression in T-B lymphoblast hybrids. *Immunogenetics,* 21**,** 235-46.

SANGIOLO, D., LESNIKOVA, M., NASH, R. A., JENSEN, M. C., NIKITINE, A., KIEM, H.-P. & GEORGES, G. E. 2007. Lentiviral vector conferring resistance to mycophenolate mofetil and sensitivity to ganciclovir for in vivo T-cell selection. *Gene Therapy,* 14**,** 1549-1554.

SAVOLDO, B., CUBBAGE, M. L., DURETT, A. G., GOSS, J., HULS, M. H., LIU, Z., TERESITA, L., GEE, A. P., LING, P. D., BRENNER, M. K., HESLOP, H. E. & ROONEY, C. M. 2002. Generation of EBV-specific CD4+ cytotoxic T cells from virus naive individuals. *Journal of Immunology,* 168**,** 909-18.

SAVOLDO, B., GOSS, J., LIU, Z., HULS, M. H., DOSTER, S., GEE, A. P., BRENNER, M. K., HESLOP, H. E. & ROONEY, C. M. 2001. Generation of autologous Epstein-Barr virus-specific cytotoxic T cells for adoptive immunotherapy in solid organ transplant recipients. *Transplantation,* 72**,** 1078-86.

SAVOLDO, B., GOSS, J. A., HAMMER, M. M., ZHANG, L., LOPEZ, T., GEE, A. P., LIN, Y. F., QUIROS-TEJEIRA, R. E., REINKE, P., SCHUBERT, S., GOTTSCHALK, S., FINEGOLD, M. J., BRENNER, M. K., ROONEY, C. M. & HESLOP, H. E. 2006. Treatment of solid organ transplant recipients with autologous Epstein Barr virus-specific cytotoxic T lymphocytes (CTLs). *Blood,* 108**,** 2942-9.

SCHLUNS, K. S., KIEPER, W. C., JAMESON, S. C. & LEFRANCOIS, L. 2000. Interleukin-7 mediates the homeostasis of naïve and memory CD8 T cells in vivo. *Nature Immunology,* 1**,** 426-432.

SCHUB, A., SCHUSTER, I. G., HAMMERSCHMIDT, W. & MOOSMANN, A. 2009. CMV-specific TCR-transgenic T cells for immunotherapy. *Journal of Immunology,* 183**,** 6819-30.

SCOTT, J. W., HAWLEY, S. A., GREEN, K. A., ANIS, M., STEWART, G., SCULLION, G. A., NORMAN, D. G. & HARDIE, D. G. 2004. CBS domains form energy-sensing modules whose binding of adenosine ligands is

disrupted by disease mutations. *Journal of Clinical Investigation,* 113**,** 274-84.

SEEGMILLER, J. E., ROSENBLOOM, F. M. & KELLEY, W. N. 1967. Enzyme defect associated with a sex-linked human neurological disorder and excessive purine synthesis. *Science,* 155**,** 1682-4.

SENDA, M., DELUSTRO, B., EUGUI, E. & NATSUMEDA, Y. 1995. Mycophenolic acid, an inhibitor of IMP dehydrogenase that is also an immunosuppressive agent, suppresses the cytokine-induced nitric oxide production in mouse and rat vascular endothelial cells. *Transplantation,* 60**,** 1143-1148.

SENDA, M. & NATSUMEDA, Y. 1994. Tissue-differential expression of two distinct genes for human IMP dehydrogenase (E.C.1.1.1.205). *Life sciences,* 54**,** 1917-1926.

SHAPIRA, M. Y., HIRSHFELD, E., WEISS, L., ZEIRA, M., KASIR, J., OR, R., RESNICK, I. B. & SLAVIN, S. 2004. Mycophenolate mofetil does not suppress the graft-versus-leukemia effect or the activity of lymphokine-activated killer (LAK) cells in a murine model. *Cancer Immunology, Immunotherapy,* 54**,** 383-388.

SHAPIRO, R. S., MCCLAIN, K., FRIZZERA, G., GAJL-PECZALSKA, K. J., KERSEY, J. H., BLAZAR, B. R., ARTHUR, D. C., PATTON, D. F., GREENBERG, J. S., BURKE, B. & ET AL. 1988. Epstein-Barr virus associated B cell lymphoproliferative disorders following bone marrow transplantation. *Blood,* 71**,** 1234-43.

SHASTRI, N. & GONZALEZ, F. 1993. Endogenous generation and presentation of the ovalbumin peptide/Kb complex to T cells. *The Journal of immunology,* 150**,** 2724-2736.

SHAW, L. M. & NOWAK, I. 1995. Mycophenolic acid: measurement and relationship to pharmacologic effects. *Therapeutic drug monitoring,* 17**,** 685.

SHERMAN, M. L., DATTA, R., HALLAHAN, D. E., WEICHSELBAUM, R. R. & KUFE, D. W. 1991. Regulation of tumor necrosis factor gene expression by ionizing radiation in human myeloid leukemia cells and peripheral blood monocytes. *Journal of Clinical Investigation,* 87**,** 1794-7.

SHERRITT, M. A., BHARADWAJ, M., BURROWS, J. M., MORRISON, L. E., ELLIOTT, S. L., DAVIS, J. E., KEAR, L. M., SLAUGHTER, R. E., BELL, S. C., GALBRAITH, A. J., KHANNA, R. & MOSS, D. J. 2003. Reconstitution of the latent T-lymphocyte response to Epstein-Barr virus is coincident with long-term recovery from posttransplant lymphoma after adoptive immunotherapy. *Transplantation,* 75**,** 1556-60.

SHIMIZU, J., YAMAZAKI, S. & SAKAGUCHI, S. 1999. Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. *Journal of Immunology,* 163**,** 5211-8.

SINTCHAK, M. D., FLEMING, M. A., FUTER, O., RAYBUCK, S. A., CHAMBERS, S. P., CARON, P. R., MURCKO, M. A. & WILSON, K. P. 1996. Structure and mechanism of inosine monophosphate dehydrogenase in complex with the immunosuppressant mycophenolic acid. *Cell,* 85**,** 921.

SOLLINGER, H. W. 1995. Mycophenolate mofetil for the prevention of acute rejection in primary cadaveric renal allograft recipients. U.S. Renal Transplant Mycophenolate Mofetil Study Group. *Transplantation,* 60**,** 225-32.

SOLLINGER, H. W., DEIERHOI, M. H., BELZER, F. O., DIETHELM, A. G. & KAUFFMAN, R. S. 1992. RS-61443--a phase I clinical trial and pilot rescue study. *Transplantation,* 53**,** 428-32.

STANBURY, R. M. & GRAHAM, E. M. 1998. Systemic corticosteroid therapy--side effects and their management. *Br J Ophthalmol,* 82**,** 704-8.

STANISLAWSKI, T., VOSS, R. H., LOTZ, C., SADOVNIKOVA, E., WILLEMSEN, R. A., KUBALL, J., RUPPERT, T., BOLHUIS, R. L., MELIEF, C. J., HUBER, C., STAUSS, H. J. & THEOBALD, M. 2001. Circumventing tolerance to a human MDM2-derived tumor antigen by TCR gene transfer. *Nature Immunology,* 2**,** 962-70.

STEVEN, N. M. 1997. Epstein-Barr virus latent infection in vivo. *Reviews in Medical Virology,* 7**,** 97-106.

STEVENS, S. J., VERSCHUUREN, E. A., PRONK, I., VAN DER BIJ, W., HARMSEN, M. C., THE, T. H., MEIJER, C. J., VAN DEN BRULE, A. J. & MIDDELDORP, J. M. 2001. Frequent monitoring of Epstein-Barr virus DNA load in unfractionated whole blood is essential for early detection of posttransplant lymphoproliferative disease in high-risk patients. *Blood,* 97**,** 1165-71.

STOREK, J. 2008. Immunological reconstitution after hematopoietic cell transplantation - its relation to the contents of the graft. *Expert Opin Biol Ther,* 8**,** 583-97.

STRALEY, S. C. & HARMON, P. A. 1984. Growth in mouse peritoneal macrophages of Yersinia pestis lacking established virulence determinants. *Infection and Immunity,* 45**,** 649-54.

STRIEPEN, B., PRUIJSSERS, A. J., HUANG, J., LI, C., GUBBELS, M. J., UMEJIEGO, N. N., HEDSTROM, L. & KISSINGER, J. C. 2004. Gene transfer in the evolution of parasite nucleotide biosynthesis. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 3154-9.

SURH, C. D. & SPRENT, J. 2000. Homeostatic T cell proliferation: how far can T cells be activated to self-ligands? *Journal of Experimental Medicine,* 192**,** F9-F14.

SUTMULLER, R. P., VAN DUIVENVOORDE, L. M., VAN ELSAS, A., SCHUMACHER, T. N., WILDENBERG, M. E., ALLISON, J. P., TOES, R.

E., OFFRINGA, R. & MELIEF, C. J. 2001. Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. *Journal of Experimental Medicine,* 194**,** 823-32.

SUZUKI, S., KIMURA, T., ANDO, K., SAWADA, M. & TAMURA, G. 1969. Antitumor activity of mycophenolic acid. *Journal of Antibiotics,* 22**,** 297-302.

SWEENEY, M. J., HOFFMAN, D. H. & ESTERMAN, M. A. 1972. Metabolism and biochemistry of mycophenolic acid. *Cancer Research,* 32**,** 1803-9.

SWINNEN, L. J. 2000. Diagnosis and treatment of transplant-related lymphoma. *Annals of Oncology,* 11 Suppl 1**,** 45-8.

SYLWESTER, A. W., MITCHELL, B. L., EDGAR, J. B., TAORMINA, C., PELTE, C., RUCHTI, F., SLEATH, P. R., GRABSTEIN, K. H., HOSKEN, N. A., KERN, F., NELSON, J. A. & PICKER, L. J. 2005. Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. *Journal of Experimental Medicine,* 202**,** 673-85.

TAN, J. T. 2002. Interleukin (IL)-15 and IL-7 Jointly Regulate Homeostatic Proliferation of Memory Phenotype CD8+ Cells but Are Not Required for Memory Phenotype CD4+ Cells. *Journal of Experimental Medicine,* 195**,** 1523-1532.

TANABE, M., TODO, S., MURASE, N., IRISH, W., MIYAZAWA, H., FUJISAKI, S. & STARZL, T. E. 1994. Combined immunosuppressive therapy with low dose FK506 and antimetabolites in rat allogeneic heart transplantation. *Transplantation,* 58**,** 23-7.

TANNER, J. E. & ALFIERI, C. 2001. The Epstein-Barr virus and post-transplant lymphoproliferative disease: interplay of immunosuppression, EBV, and the immune system in disease pathogenesis. *Transplant Infectious Disease,* 3**,** 60-9.

THE TRICONTINENTAL MYCOPHENOLATE MOFETIL RENAL TRANSPLANTATION STUDY GROUP 1996. A blinded, randomized clinical trial of mycophenolate mofetil for the prevention of acute rejection in cadaveric renal transplantation. . *Transplantation,* 61**,** 1029-37.

THOMAS, E. C., GUNTER, J. H., WEBSTER, J. A., SCHIEBER, N. L., OORSCHOT, V., PARTON, R. G. & WHITEHEAD, J. P. 2012. Different Characteristics and Nucleotide Binding Properties of Inosine Monophosphate Dehydrogenase (IMPDH) Isoforms. *PloS One,* 7.

THOMAS, S., XUE, S. A., CESCO-GASPERE, M., SAN JOSE, E., HART, D. P., WONG, V., DEBETS, R., ALARCON, B., MORRIS, E. & STAUSS, H. J. 2007. Targeting the Wilms tumor antigen 1 by TCR gene transfer: TCR variants improve tetramer binding but not the function of gene modified human T cells. *Journal of Immunology,* 179**,** 5803-10.

THOMPSON, M. P. & KURZROCK, R. 2004. Epstein-Barr virus and cancer. *Clinical Cancer Research,* 10**,** 803-21.

TOUBIANA, J., ROSSI, A. L., GRIMALDI, D., BELAIDOUNI, N., CHAFEY, P., CLARY, G., COURTINE, E., PENE, F., MIRA, J. P., CLAESSENS, Y. E. & CHICHE, J. D. 2011. IMPDHII Protein Inhibits Toll-like Receptor 2-mediated Activation of NF- B. *Journal of Biological Chemistry,* 286**,** 23319-23333.

TRAMSEN, L., SCHMIDT, S., ROEGER, F., SCHUBERT, R., SALZMANN-MANRIQUE, E., LATGE, J. P., KLINGEBIEL, T. & LEHRNBECHER, T. 2014. Immunosuppressive compounds exhibit particular effects on functional properties of human anti-Aspergillus Th1 cells. *Infect Immun,* 82**,** 2649-56.

TREDGER, J. M., BROWN, N. W., ADAMS, J., GONDE, C. E., DHAWAN, A., RELA, M. & HEATON, N. 2004. Monitoring mycophenolate in liver transplant recipients: Toward a therapeutic range. *Liver Transplantation,* 10**,** 492-502.

TRIVEDI, D., WILLIAMS, R. Y., O'REILLY, R. J. & KOEHNE, G. 2005. Generation of CMV-specific T lymphocytes using protein-spanning pools of pp65-derived overlapping pentadecapeptides for adoptive immunotherapy. *Blood,* 105**,** 2793-801.

TSANG, J. Y., TANRIVER, Y., JIANG, S., XUE, S. A., RATNASOTHY, K., CHEN, D., STAUSS, H. J., BUCY, R. P., LOMBARDI, G. & LECHLER, R. 2008. Conferring indirect allospecificity on CD4+CD25+ Tregs by TCR gene transfer favors transplantation tolerance in mice. *Journal of Clinical Investigation,* 118**,** 3619-28.

VAN BRUGGEN, M. C., WALGREEN, B., RIJKE, T. P. & BERDEN, J. H. 1998. Attenuation of murine lupus nephritis by mycophenolate mofetil. *Journal of the American Society of Nephrology : JASN,* 9**,** 1407-1415.

VAN GELDER, T., LE MEUR, Y., SHAW, L. M., OELLERICH, M., DENOFRIO, D., HOLT, C., HOLT, D. W., KAPLAN, B., KUYPERS, D., MEISER, B., TOENSHOFF, B. & MAMELOK, R. D. 2006. Therapeutic drug monitoring of mycophenolate mofetil in transplantation. *Therapeutic Drug Monitoring,* 28**,** 145-54.

VAN GELDER, T., SILVA, H. T., DE FIJTER, J. W., BUDDE, K., KUYPERS, D., TYDEN, G., LOHMUS, A., SOMMERER, C., HARTMANN, A., LE MEUR, Y., OELLERICH, M., HOLT, D. W., TONSHOFF, B., KEOWN, P., CAMPBELL, S. & MAMELOK, R. D. 2008. Comparing mycophenolate mofetil regimens for de novo renal transplant recipients: the fixed-dose concentration-controlled trial. *Transplantation,* 86**,** 1043-51.

VAN HEST, R. M., MATHOT, R. A., PESCOVITZ, M. D., GORDON, R., MAMELOK, R. D. & VAN GELDER, T. 2006. Explaining variability in mycophenolic acid exposure to optimize mycophenolate mofetil dosing: a population pharmacokinetic meta-analysis of mycophenolic acid in renal transplant recipients. *J Am Soc Nephrol,* 17**,** 871-80.

VAN HEST, R. M., VAN GELDER, T., VULTO, A. G. & MATHOT, R. A. 2005. Population pharmacokinetics of mycophenolic acid in renal transplant recipients. *Clin Pharmacokinet,* 44**,** 1083-96.

VEENSTRA, D. L., BEST, J. H., HORNBERGER, J., SULLIVAN, S. D. & HRICIK, D. E. 1999. Incidence and long-term cost of steroid-related side effects after renal transplantation. *Am J Kidney Dis,* 33**,** 829-39.

VO, A. A., PENG, A., TOYODA, M., KAHWAJI, J., CAO, K., LAI, C. H., REINSMOEN, N. L., VILLICANA, R. & JORDAN, S. C. 2010. Use of intravenous immune globulin and rituximab for desensitization of highly HLA-sensitized patients awaiting kidney transplantation. *Transplantation,* 89**,** 1095-102.

WALKER, L. S., CHODOS, A., EGGENA, M., DOOMS, H. & ABBAS, A. K. 2003. Antigen-dependent proliferation of CD4+ CD25+ regulatory T cells in vivo. *Journal of Experimental Medicine,* 198**,** 249-58.

WALTER, E. A., GREENBERG, P. D., GILBERT, M. J., FINCH, R. J., WATANABE, K. S., THOMAS, E. D. & RIDDELL, S. R. 1995. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. *New England Journal of Medicine,* 333**,** 1038-1044.

WANG, J., ZEEVI, A., WEBBER, S., GIRNITA, D. M., ADDONIZIO, L., SELBY, R., HUTCHINSON, I. V. & BURCKART, G. J. 2007. A novel variant L263F in human inosine 5'-monophosphate dehydrogenase 2 is associated with diminished enzyme activity. *Pharmacogenetics and genomics,* 17**,** 283-290.

WEBER, G., HAGER, J. C., LUI, M. S., PRAJDA, N., TZENG, D. Y., JACKSON, R. C., TAKEDA, E. & EBLE, J. N. 1981. Biochemical programs of slowly and rapidly growing human colon carcinoma xenografts. *Cancer Res,* 41**,** 854-9.

WEBER, L. T., SHIPKOVA, M., ARMSTRONG, V. W., WAGNER, N., SCHÜTZ, E., MEHLS, O., ZIMMERHACKL, L. B., OELLERICH, M. & TÖNSHOFF, B. 2002. Comparison of the Emit immunoassay with HPLC for therapeutic drug monitoring of mycophenolic acid in pediatric renal-transplant recipients on mycophenolate mofetil therapy. *Clinical chemistry,* 48**,** 517-525.

WEBSTER, H. K. & WHAUN, J. M. 1982. Antimalarial properties of bredinin. Prediction based on identification of differences in human host-parasite purine metabolism. *Journal of Clinical Investigation,* 70**,** 461-9.

WEGENER, A. M., LETOURNEUR, F., HOEVELER, A., BROCKER, T., LUTON, F. & MALISSEN, B. 1992. The T cell receptor/CD3 complex is composed of at least two autonomous transduction modules. *Cell,* 68**,** 83-95.

WEIGEL, G., GRIESMACHER, A., KARIMI, A., ZUCKERMANN, A. O., GRIMM, M. & MUELLER, M. M. 2002. Effect of mycophenolate mofetil therapy on

lymphocyte activation in heart transplant recipients. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation,* 21**,** 1074-1079.

WILKIE, G. M., TAYLOR, C., JONES, M. M., BURNS, D. M., TURNER, M., KILPATRICK, D., AMLOT, P. L., CRAWFORD, D. H. & HAQUE, T. 2004. Establishment and characterization of a bank of cytotoxic T lymphocytes for immunotherapy of epstein-barr virus-associated diseases. *Journal of Immunotherapy,* 27**,** 309-316.

WILLIAMS, R. H., LIVELY, D. H., DELONG, D. C., CLINE, J. C. & SWEENY, M. J. 1968. Mycophenolic acid: antiviral and antitumor properties. *Journal of Antibiotics,* 21**,** 463.

WILSON, K., BERENS, R. L., SIFRI, C. D. & ULLMAN, B. 1994. Amplification of the inosinate dehydrogenase gene in Trypanosoma brucei gambiense due to an increase in chromosome copy number. *Journal of Biological Chemistry,* 269**,** 28979-87.

WILSON, K., COLLART, F. R., HUBERMAN, E., STRINGER, J. R. & ULLMAN, B. 1991. Amplification and molecular cloning of the IMP dehydrogenase gene of Leishmania donovani. *Journal of Biological Chemistry,* 266**,** 1665-71.

XING, T., HUANG, L., YU, Z., ZHONG, L., WANG, S. & PENG, Z. 2013. Comparison of steroid-free immunosuppression and standard immunosuppression for liver transplant patients with hepatocellular carcinoma. *PLoS One,* 8**,** e71251.

XUE, S. A., GAO, L., HART, D., GILLMORE, R., QASIM, W., THRASHER, A., APPERLEY, J., ENGELS, B., UCKERT, W., MORRIS, E. & STAUSS, H. 2005. Elimination of human leukemia cells in NOD/SCID mice by WT1-TCR gene-transduced human T cells. *Blood,* 106**,** 3062-7.

XUN, C. Q., THOMPSON, J. S., JENNINGS, C. D., BROWN, S. A. & WIDMER, M. B. 1994. Effect of total body irradiation, busulfan-cyclophosphamide, or cyclophosphamide conditioning on inflammatory cytokine release and development of acute and chronic graft-versus-host disease in H-2-incompatible transplanted SCID mice. *Blood,* 83**,** 2360-7.

YAM, P., JENSEN, M., AKKINA, R., ANDERSON, J., VILLACRES, M., WU, J., ZAIA, J. & YEE, J. 2006. Ex Vivo Selection and Expansion of Cells Based on Expression of a Mutated Inosine Monophosphate Dehydrogenase 2 after HIV Vector Transduction: Effects on Lymphocytes, Monocytes, and CD34+ Stem Cells. *Molecular Therapy,* 14**,** 236-244.

YAMANI, M. H., STARLING, R. C., GOORMASTIC, M., VAN LENTE, F., SMEDIRA, N., MCCARTHY, P. & YOUNG, J. B. 2000. The impact of routine mycophenolate mofetil drug monitoring on the treatment of cardiac allograft rejection. *Transplantation,* 69**,** 2326-30.

YOKOYAMA, I., HAYASHI, S., KOBAYASHI, T., NEGITA, M., YASUTOMI, M., UCHIDA, K. & TAKAGI, H. 1995. Immunosuppressive drugs and their effect on experimental tumor growth. *Transpl Int,* 8**,** 251-5.

YU, C. C., YANG, C. W., WU, M. S., KO, Y. C., HUANG, C. T., HONG, J. J. & HUANG, C. C. 2001. Mycophenolate mofetil reduces renal cortical inducible nitric oxide synthase mRNA expression and diminishes glomerulosclerosis in MRL/lpr mice. *Journal of Laboratory and Clinical Medicine,* 138**,** 69-77.

YU, J., LEMAS, V., PAGE, T., CONNOR, J. D. & YU, A. L. 1989. Induction of erythroid differentiation in K562 cells by inhibitors of inosine monophosphate dehydrogenase. *Cancer Res,* 49**,** 5555-60.

ZANDVLIET, M. L., VAN LIEMPT, E., JEDEMA, I., KRUITHOF, S., KESTER, M. G., GUCHELAAR, H. J., FALKENBURG, J. H. & MEIJ, P. 2011. Simultaneous isolation of CD8(+) and CD4(+) T cells specific for multiple viruses for broad antiviral immune reconstitution after allogeneic stem cell transplantation. *Journal of Immunotherapy,* 34**,** 307-19.

ZENG, L., BLAIR, E. Y., NATH, C. E., SHAW, P. J., EARL, J. W., STEPHEN, K., MONTGOMERY, K., COAKLEY, J. C., HODSON, E., STORMON, M. & MCLACHLAN, A. J. 2010. Population pharmacokinetics of mycophenolic acid in children and young people undergoing blood or marrow and solid organ transplantation. *Br J Clin Pharmacol,* 70**,** 567-79.

ZHANG, Y., LOUBOUTIN, J. P., ZHU, J., RIVERA, A. J. & EMERSON, S. G. 2002. Preterminal host dendritic cells in irradiated mice prime CD8+ T cell-mediated acute graft-versus-host disease. *Journal of Clinical Investigation,* 109**,** 1335-44.

ZHOU, F., ROUSE, B. T. & HUANG, L. 1992. Prolonged survival of thymoma-bearing mice after vaccination with a soluble protein antigen entrapped in liposomes: a model study. *Cancer Res,* 52**,** 6287-91.

ZUCKERMANN, A., PLONER, M., KEZIBAN, U., CZERNY, M., WOLNER, E., LAUFER, G. & GRIMM, M. 2001. Benefit of mycophenolate-mofetil (MMF) in patients with cyclosporine (CYA) induced nephropathy after cardiac transplantation. *Journal of Heart and Lung Transplantation,* 20**,** 163.